Investigating Potential Effects of Dengue Virus Infection and Pre-exposure to DEET on *Aedes aegypti* Behaviors

by

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Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2016

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

APPROVAL OF THE DOCTORAL DISSERTATION IN THE
EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "Investigating Potential Effects of Dengue Virus Infection and Pre-exposure to DEET on *Aedes aegypti* Behaviors"

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February 5, 2016

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ACKNOWLEDGMENTS

First, I would like to thank all members of my committee, past and present, for their willingness to share their knowledge and wisdom: Dr. Jittawadee Murphy, Dr. Douglas Merrell, Dr. Michael Turell, Dr. Michelle Colacicco-Mayhugh, Dr. Ann Stewart, Dr. Cara Olsen, Dr. Edgar Rowton, Dr. John Grieco, and Dr. Nicole Achee. My appreciation also goes to Dr. Christopher Broder who always helped and supported me during difficult times. I also would like to thank the GEO and PMB administration teams.

Thank you to my wonderful classmates: Holly, Angela, Leah, and Joe; with whom I have the honor of sharing the journey of getting a doctorate degree. I would also like to thank other members of my lab: Suppaluck Polsomboon and Wilawan Thipmontree for their generous help.

Thanks to Dr. Wei-Mei Ching and Dr. Chien-Chung Chao at the Naval Medical Research Center for their generosity in supporting me with laboratory equipment, space, discussion, and many other types of kindness that are impossible to all be listed.

Many thanks to my dear friends Susana Widjaja and Sri Hadiwidjojo who have been my strongest supports. I would not be able to finish this without your continuous kindness, friendship, and support. Also, my appreciation to my good friend Carlos Mena for your valuable life advice.

I would like to dedicate my biggest thanks to my family for supporting me throughout this journey and for their kind patience and understanding that I cannot be there for them when they need me the most: my Dad, Alex Sugiharto, who is always there for me and calls me everyday; my Mom, Naniek Retnaning Tias, who is

shouldering the entire family burden while I am here; my dearest sisters, Aisyah Ningtyas Sugiharto and Melysyah Ningtyas Sugiharto, who always want me to be happy and support me unconditionally.

DEDICATION

In loving memory of my dearest Grandmother, Susilowati. I regret not being able to be with you in your final moments because of my qualifying exam. This is something that I will regret for the rest of my life. I love you and miss you every single day.

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Victor A. Sugiharto

May 20th 2016

ABSTRACT

Investigating Potential Effects of Dengue Virus Infection and Pre-exposure to DEET on *Aedes aegypti* Behaviors

Victor A. Sugiharto, Ph.D., 2016

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Other than being a nuisance, mosquito bites can potentially transmit pathogens, to include malaria parasites and dengue virus (DENV), which can cause severe diseases and mortality. Therefore, reducing mosquito-human contact is an important step to prevent diseases. Mosquito behaviors are heavily influenced by chemical cues in the surrounding environment that are perceived through the mosquito olfactory system. This knowledge has been harnessed to human advantages, such as in the development of some traps and repellent chemicals. However, mosquito behaviors have been reported to change following pathogen infection or previous chemical exposure. This behavioral change can potentially diminish the value of preventive measures, such as the widely used repellent, DEET. In this study, we assessed potential behavioral change that might stem from DENV infection or DEET pre-exposure as a means to understand how these factors might affect the efficacy of DEET as a preventive tool for public health.

In our first aim, we evaluated if infection by DENV-1 could alter the behavioral response of *Aedes aegypti* mosquitoes to DEET. Using the high throughput screening system (HITSS) chamber, we subjected three different groups of mosquitoes (DENV-1-injected, diluent-injected, and uninjected) to behavioral tests in order to identify any temporal and concentration dependent behavioral changes from DENV-1 infection. We found no effect of DENV-1 infection on the irritancy behavioral response of *Ae. aegypti* to DEET. From the public health perspective, this result should be seen as an encouraging one as it provides evidence of DEET efficacy in inducing irritancy in DENV-1-infected, as well as uninfected mosquitoes. However, additional studies involving other aspects of mosquito behavior, other arthropod-borne viruses (arboviruses), and other chemicals are necessary to provide the full answer on the effect of infection on vector behavior.

In our second study, we assessed the effect of prior exposure to DEET on the subsequent blood-feeding behavior of *Ae. aegypti* mosquitoes. The mosquitoes were exposed to DEET for 10 minutes and either immediately given a blood meal source or incubated for selected time intervals before being given a blood meal source. We then measured landing, probing, and blood level engorgement of these mosquitoes and compared them to the ethanol-exposed mosquitoes that acted as control cohort. We found that prior DEET exposure did not alter the landing and probing behavior at any concentration or incubation time tested. However, pre-exposure to 0.14 or 0.16% DEET reduced the overall mosquito blood engorgement level within 24 hours post exposure, with the reduction at 3 and 6 hours post exposure being statistically significant. This result raises concern that prior exposure to DEET could potentially increase the vectorial

capacity of mosquitoes because incomplete blood meal intake has been associated with increased refeeding.

There are still many aspects of pathogen infection and chemical exposure with regard to their effects on insect behavior that have not been explored. It may be necessary in the future to test repellents against infected mosquitoes in order to determine if they behave differently compared to their uninfected counterparts. In addition, further research exploring the blood-feeding behavior of infected mosquitoes that have been previously exposed to repellents is necessary in order to ensure that the usage of repellent does not actually do more harm than good. This will contribute to the improvement of this public health tool in combating specific mosquito-borne diseases in a more effective manner.

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CHAPTER 1: General Introduction

VECTOR-BORNE DISEASES

Vector-borne diseases are caused by pathogens that are spread mainly by another living organism or vector, mostly through the vectors' bites (19; 141). Out of all disease vectors, mosquitoes are the most significant one. Malaria alone was estimated by the World Health Organization (WHO) to be responsible for more than half of the total mortality from all vector-borne diseases. This is followed by the combined total mortality from mosquito-borne virus infections, such as dengue virus (DENV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV) (141).

The dynamics of mosquito-borne pathogens are very complex (19). Yet, the main principle is the requirement for contact in the form of blood-feeding on the host. Therefore, reducing vector-host contact is an important part of reducing the incidence rate. If a person infected with one of these pathogens can reduce their contact with mosquitoes, there will be less pathogen-infected mosquitoes able to transmit the pathogen, and if susceptible hosts can reduce their contact with mosquitoes, they are at reduced risk of being bitten by an infectious mosquito. A repellent is an excellent example of a prevention method to reduce vector-host contact. However, previous studies have shown that pathogen infection or previous chemical exposure can alter a vector subsequent behavior, thus potentially diminishing the value of a repellent as preventive measure (100-102; 122; 135). In this study, we assessed potential behavioral change that might stem from DENV-1 infection or N,N-Diethyl-meta-toluamide (DEET) pre-exposure as a

means to assess how these factors might affect the efficacy of DEET as a preventive tool for public health.

MOSQUITOES

Mosquitoes (Class Insecta, Order Diptera, Family Culicidae) can be found all over the world except in perpetually frozen areas. Mosquitoes undergo full metamorphosis with 4 life stages: egg, larva, pupa, and adult. Eggs are deposited in aquatic environments either directly on the water or areas that will be submerged by water. The larvae feed on detritus and have four instar stages. The temperature and nutrition ingested during larval stages determine the time required for the larvae to molt into pupae. Once they molt into pupae, they do not require any food. Depending on temperature, usually after a couple of days of pupation, the pupae will rise to the surface and eclose into an adult or imago (2).

Male adult mosquitoes usually eclose earlier than their female counterparts. The male genitalia need about one day to rotate and be ready for mating. Both genders can actually subsist just by feeding on nectar or another sugar source. The females of mosquito species, except those in the genus *Toxorhynchites*, require a blood meal for oogenesis, causing them to be well-known as a biting nuisance and also vectors for various infectious diseases. Once a female mosquito acquires a blood meal, she will undergo vitellogenesis that switches the behavior from blood-seeking to egg-ovipositing. Once the eggs are oviposited, the female mosquitoes are ready to obtain another blood meal (2).

Among numerous diseases transmitted by mosquitoes, malaria and dengue fever are the two most important and prevalent mosquito-borne diseases in the world. Although

the number of malaria cases is declining worldwide due to the success of various vector control efforts, the number of infections with DENV is increasing (Figure 1) (139; 142). Various other arthropod-borne viruses (arboviruses) are also transmitted via mosquito bites, to include chikungunya virus (CHIKV), YFV, and West Nile virus. When an infectious mosquito bites a suitable host, the pathogen-containing saliva is injected into the host transferring the disease pathogen. The capability of a mosquito species to become a vector for a disease is called vector competence, while the efficiency of it as a vector is called vectorial capacity (91).

Aedes aegypti

Aedes aegypti is commonly known as the yellow fever mosquito. This is due to the fact that they were notorious as the vector of the YFV. Currently, *Ae. aegypti* is more well-known as the vector of DENV and CHIKV (126). Due to its importance as a human disease vector and ease of laboratory breeding compared to other mosquito vectors, *Ae. aegypti* has been used widely as the model for entomological research (27; 89).

This mosquito is believed to have originated from North Sub-Saharan Africa (15). One of its subspecies, *Ae. aegypti formosus*, retains more of the ancestral sylvatic traits, such as choosing tree holes as an oviposition site and preferring a nonhuman blood source (15; 99). The increasing geographic movement by European settlers beginning in the 16th century introduced the *Ae. aegypti* to other parts of the world; the mosquito hitched a ride in ship water containers (15; 66). The mosquitoes were introduced to the Americas through slave trade, concurrently introducing the YFV into the New World. The introduction of this mosquito to Asia Pacific came later at around the 19th century, which then subsequently allowed for urban cases of DENV in Asia Pacific (99).

The *Ae. aegypti* mosquito has adapted well to human living conditions (15; 99). It is very anthrophilic and has also adapted to be a container breeder, which makes it an excellent urban vector for viruses such as DENV, YFV, CHIKV, and Zika virus (15; 99). Currently, the *Ae. aegypti* mosquito can live between 40^{o} N and 40^{o} S latitudes (Figure 2) (78).

Aedes albopictus

Aedes albopictus is also a competent vector for CHIKV and DENV. It is a more weather tolerant species and can live in wider area (between 42^{o} N and 42^{o} S latitudes) than *Ae. aegypti* (Figure 3) (39; 58). This mosquito species has replaced *Ae. aegypti* as the dominant species in many parts of the world, including the continental United States, since its introduction in imported used tires in 1985 (8; 58; 63). Fortunately, *Ae. albopictus* is not as anthropophilic as *Ae. aegypti* (76; 120). Moreover, although they are more prone to become infected, *Ae. albopictus* were demonstrated to be less likely to become infectious (138). However, based on the fact that *Ae. aegypti* mosquitoes were able to adapt to live in very close proximity with human, and the fact that the arboviruses then also adapted to use *Ae. aegypti* as their new vector, it is possible that *Ae. albopictus* may eventually become an important vector of various disease pathogens in the future (29; 67; 98; 130).

Olfactory System

Mosquito behavior is influenced by various chemical signals recognized by their olfactory system (104). Chemicals emitted by humans and other vertebrates, such as lactic acid, carbon dioxide, and octenol, are detected and allow mosquitoes to locate their hosts. In mosquitoes, the main olfactory organs are the antennae and maxillary palps,

which are covered with specialized hairs, called sensilla, that have sensory functions. Each sensillum has a multiporous structure that allows chemical molecules to enter and one to five olfactory receptor neurons (ORN). There are two important groups of olfactory proteins present in an ORN: odorant binding proteins (OBP) and olfactory receptors. The OBP binds and transports molecules through the aqueous environment of the sensillum. There are three groups of olfactory receptors: odorant receptors (OR), gustatory receptors (GR), and ionotrophic receptors (IR). The OR are believed to function in detecting general odorants. For it to function, an obligate OR called Orco must form a dimer with another OR, which then creates a ligand-gated ion channel. Gustatory receptors works in detecting CO_2; mosquito that lacks the GR3 protein are unresponsive to CO_2 stimuli. Ionotrophic receptor is believed to detect acids and amines (48; 124). The axons of ORN extend into the antennal lobe of the mosquito brain, which is composed of multiple glomeruli. Signals from activated ORNs are delivered to the corresponding glomerulus inside the antennal lobe and the projection neuron of this lobe delivers the signal further to the higher brain area (48).

DENGUE

Dengue viruses are enveloped viruses of the genus *Flavivirus* and the family *Flaviviridae*. They have a 11kb single-stranded positive RNA as its genetic material. Upon infection, the RNA is readily translatable into a polyprotein that is further cleaved by both host and viral proteases into three structural proteins (capsid, pre-membrane, envelop) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). There are currently four circulating serotypes, aptly named DENV-1, -2, -3, and -4. The virus was first isolated in 1943 in Japan (DENV-1), followed by isolation in 1945 in

Hawaii (DENV-2), and in 1953 in the Philippines and Thailand (DENV-3 and -4, respectively) (88). These serotypes have about 65% sequence identity in their genomes (53).

Dengue virus is currently considered by the WHO to be the most important arbovirus in the world (139). Infection with the virus has manifestations that range from asymptomatic infection, mild undifferentiated fever, dengue fever, to fatal dengue hemorrhagic fever/dengue shock syndrome. Dengue fever symptoms consist of fever, headache, nausea, joint and muscle pain, and thrombocytopenia. In severe cases, the thrombocytopenia becomes severe and hemorrhage occurs, which can lead to shock and death (139).

Infection with one serotype does not confer life-long protection to subsequent heterotypic infection. Instead, the protection against the other serotypes only lasts for two to three months. Furthermore, it has been shown that if a secondary heterotypic infection occurs, the clinical manifestation may be more severe. This is hypothesized to be antibody dependent enhancement where the sub-neutralizing antibody helps the virus gain entrance into susceptible cells (52).

The *Aedes aegypti* mosquito is the principal vector for DENV. Through an infected blood meal, the virus may infect and replicate in the mosquito midgut cells until it “escapes” to infect various body parts of the mosquito, including brain, malphigian tubules, and ovaries, and possibly the salivary glands (known as a disseminated infection). Once it infects the salivary glands and is secreted in the saliva, the virus can then be transmitted via mosquito probing for a blood meal (40).

Pathogenesis

Upon introduction to the human host via an infectious mosquito bite, DENV infects the Langerhans cells. As an antigen presenting cell, the Langerhans cells then travel to the nearest lymph node. The recruited monocytes and macrophages in the lymph node will then be infected and will distribute themselves through the lymphatic and vascular system. This condition is known as viremia. Some other cells, such as liver cells, endothelial cells, and stromal cells have been shown to be susceptible to infection *in vitro* and are suspected to contribute to the level of viremia. The infection causes these cells to undergo apoptosis and necrosis, which leads to the release of toxins and cytokines that then trigger coagulation disorders, to include fibrinolysis and thrombocytopenia. Combined with the high viral load and the cytokine storm, these conditions create a vascular leak that is the hallmark of dengue hemorrhagic fever/dengue shock syndrome (85).

In a secondary heterotypic infection, sub-neutralizing heterotypic antibody helps the virus infect the cells in a phenomenon called “antibody dependent enhancement.” When sub-neutralizing antibodies bind to the virus, they do not neutralize the virus. Instead the virus is taken up by monocytes or macrophages via the Fc gamma receptor; they gain entry to the cells and hijack the cellular machinery for replication. Thus, more cells may be infected and the virus may replicate to a higher titer, which causes more severe manifestation of the disease. Moreover, the activated memory T-cells from the primary infection have the preference to activate B-cells that have been primed to combat the first infecting serotype instead of activating a new subset of lymphocytes that are specific to the new infecting serotype. This phenomenon is known as “original antigenic sin.” The scheme of DENV pathogenesis can be viewed in Figure 4 (52; 85).

Epidemiology

The WHO had previously estimated that approximately 2.5 billion of the world's population is at risk for contracting DENV. There are an estimated 100 million infection cases of which 250,000-500,000 are severe (139). However, these numbers were drawn from the assumption that the infection rate was constant in the at risk population. Further exhaustive literature research and mathematical modeling by Bhatt *et al*. showed that the number was most likely to be significantly higher: 390 million infections of which 96 million cases were symptomatic infections (10). Furthermore, 36 countries that were considered to be DENV-free by the WHO and Centers for Disease Control and Prevention (CDC) potentially have underreported DENV cases that may not be apparent due to lack of adequate surveillance (14). As a subsequent infection with heterotypic DENV can increase the chance of developing more severe manifestation of the disease, Messina *et al*. suggested that the DENV epidemiological map should be type-specific (Figure 5) (88).

Because *Ae. aegypti* mosquitoes are associated with urban population and are very anthropophilic, human activities also contribute to the increased incidence of DENV. Low income and peri-urban areas have been associated with higher risk of DENV infection. International trade and human travel have also caused increasing numbers of DENV cases over the years. As most DENV infection results in asymptomatic infection, the risk of a traveler unknowingly introducing the virus to a naïve population is higher than in the symptomatic traveler (14).

Although the effect of climate change or global warming on disease transmission has not been thoroughly studied or clearly identified, it is reasonable to speculate that climate change can possibly exacerbate the spread of DENV to new areas. Increasing

global temperature has increased the geographical range where the mosquito vectors can thrive. As the global temperature increases, the area where aedine mosquitoes can survive expands (118). Moreover, increased temperature has been shown to shorten the mosquito developmental time or life cycle as well as the extrinsic incubation period of arboviruses (118; 143). Climate change also causes more severe weather conditions; higher rainfall can subsequently create more potential mosquito breeding grounds (73; 133). Increased occurrence of severe weather is also expected due to global warming. This phenomenon may lead to more human displacement or a disaster situation, which is associated with a higher incidence of infectious diseases, to include DENV, This is due to the failure of the public health system (68). However, Halstead argued that it is too simplistic to think that DENV will return to North America simply because of warming temperatures. His argument relies on the fact that the United States was able to eliminate YFV and DENV by good vector control, environmental manipulation, and change of human behavior (56).

Diagnostics and Treatment

Currently there is still no antiviral drug available against DENV. Patient treatment usually consists of careful fluid administration therapy. A good diagnostic methods are necessary to help patients receive the necessary therapy as soon as possible as this helps to reduce mortality. In addition, a better diagnostic method is useful for research purposes, either in field surveillance, vaccine development, or pathogenesis study (54).

Dengue can be diagnosed using various methods: virus isolation, viral nucleic acid detection, and serological assays that detect infectious virus, viral antigen, or the antibody against the virus (53; 54; 119). All the diagnostic methods have their own advantages and disadvantages. An ideal diagnostic method is cheap, quick, and easy

while still able to deliver supreme sensitivity and specificity. As DENV infection has multiple disease manifestations, the ability to accurately obtain a prognosis can help clinicians provide better care to the patients and put less burden on the healthcare system (54).

As with any other disease, choosing an appropriate diagnostic method based on disease manifestation phase or days after the onset of symptoms is crucial in order to ensure a correct diagnosis. Virus isolation, nucleic acid detection, and antigen detection are very useful in detecting early infection when the patient is still viremic. The selection for the appropriate antibody detection method requires more consideration. The antibody develops at later stages of infection and there may be different responses between primary and secondary infections; thus, it is more complicated (Figure 6).

Virus Isolation

Virus isolation is the gold standard for DENV diagnosis although its status quo has been challenged by virus nucleic acid detection assays (119). Originally, virus isolation was conducted by injecting the patient's serum into the brain of a suckling mouse; this method has a low sensitivity compared to the other isolation methods and is very cumbersome (54). The most sensitive virus isolation method is conducted by injecting patient samples into mosquitoes, either the nonblood-feeding *Toxorhynchites*, male *Ae. aegypti*, or male *Ae. albopictus*. However, similar to the mouse brain injection, this method requires special technique and special containment space (54). Currently, most virus isolation work is usually done using the C6/36 mosquito cell line, although mammalian cell lines such as Vero and BHK21 can be used with less efficiency (54; 119). The advantage of this method is that it actually proves the presence of the live virus in the

patient serum. Following virus culture, the presence of the virus is usually detected using an immunofluorescence assay, which can differentiate the infecting serotypes via specific monoclonal antibodies (54; 119). Virus isolation has the advantage of being able to actually obtain the virus that can be further sequenced for analyzing the genetic lineage, mutations, and origins of the virus. Unfortunately, not only is virus isolation labor intensive, it is also time consuming. Additionally, there is a very short viremia window in DENV patients when the virus titer is high enough to use this method accurately (53).

Nucleic Acid Detection

The DENV nucleic acid can be detected using the reverse transcriptase-polymerase chain reaction (RT-PCR) assay, either the conventional (gel-based) or real time system (26; 57; 62; 72; 77). This method has become more popular because it can be completed in a short time. It is also a very sensitive method and can be used for serotyping the infection. Unfortunately, similar to the virus isolation method, the patient sample has to be taken during viremia for the method to yield results. Moreover, the PCR assay is prone to false positive results if not performed carefully. Another disadvantage of this method is the high cost of reagents and equipment also the need for highly trained personnel (1; 54; 119).

Serological Assays

Serological assays can be modified to detect the viral antigen or the antibody against the virus. One of the serological assays i. e. the enzyme-linked immunosorbent assay (ELISA) has been employed to do both tasks. This method is relatively faster and easier than the virus isolation or molecular technique (53). The NS1 ELISA detects the presence of the DENV NS1 nonstructural protein, which is abundant during infection.

Some studies suggest that the level of the NS1 protein has a positive correlation with the development of dengue hemorrhagic fever in patients (7; 79; 94). The NS1 protein has been shown to persist longer, giving a wider window for obtaining samples needed to provide accurate diagnosis (119).

The choice of an appropriate serological assay to detect antibody against DENV requires more fine-tuning than the other diagnostic methods. In primary DENV infection, IgM antibody appears first followed by the rise of IgG titer. Subsequently, the level of IgM falls to undetectable level. However, the IgG continues to persist at lower levels even after the disease has resolved. During secondary infection, the IgG titer will rise rapidly before the IgM; the IgM titer only increases slightly for a short period of time (119).

The IgM antibody-capture ELISA detects the presence of virus-specific IgM antibody that is present during the acute phase. It is a very useful method for diagnosing acute primary infection. However, its value as a diagnostic tool diminishes in secondary cases because the IgM is undetectable or only detectable in serum samples for only a few days. This fact and the high cross-reactivity of anti-DENV IgM with other Flavivirus antigens often lead to false positive results (1). Thus, this method has a large disadvantage when used in a DENV hyperendemic region where most members of the populations are not immunologically naïve to DENV (54). An ELISA method to detect IgA has also been developed. This method was shown to be better suited for diagnosing secondary infection and can be a good complement to the IgM antibody-capture ELISA (1; 127).

The detection of IgG using ELISA requires paired sera from the patient, one from the acute and one from the convalescent phase, which may not always be available. In primary DENV cases, the IgG is usually not present in the acute phase serum but will have at least a four-fold titer increase in the convalescent sera. Conversely, in secondary DENV cases, IgG is readily present in the acute phase, followed by at least a four-fold increase in the convalescent phase (26). Unfortunately, because it requires a convalescent serum, the test result often comes too late. The plaque reduction neutralization test is a very sensitive serological method that can detect the presence of serotype specific DENV IgG in patients, unfortunately it is very labor intensive and time consuming, which limits its use in laboratory or research settings (54; 109). This method also requires both the patient's acute and convalescent sera to provide a definite result.

Prevention

Vaccine development to prevent DENV infection has been complicated by various factors. Because there are four circulating serotypes and because subsequent heterotypic infection can exacerbate the disease manifestation, the vaccine needs to be able to provide protection to all four serotypes. Moreover, the vaccine should also be useable for both fully naïve and previously exposed populations (53; 103). The lack of a good animal model for DENV infection also inhibits the progress in vaccine development (22; 103).

Sanofi Pasteur was able to get their DENV vaccine, Dengvaxia®, licensed in Mexico, the Philippines, and Brazil in December 2015 to be used in subjects aged 9 and older. The data from the phase III clinical trial in Asia and America, which started in 2011, showed seroconversion against all four serotypes with varying degrees. Moreover,

the vaccine was effective in reducing hospitalization in up to 80% of the vaccinees (16; 36; 51; 103; 116; 134). The level of seroconversion against all four serotypes and the vaccine efficacy seemed to be influenced by the immune status of the vaccine recipients (36; 103; 116). The vaccine clinical trial subjects that were immunologically naïve to DENV showed lower levels of seroconversion and protection against symptomatic infection, although protection against DENV-2 remained low across the board compared to the other serotypes (36; 116).

The newly licensed vaccine Dengvaxia® is clearly a welcome addition to the limited arsenal in the fight against DENV. However, the limited data and availability of Dengvaxia® combined with no specific treatment available to prevent or cure DENV infection suggest that the prevention methods that reduce vector and human contact are still crucial in order to bring down the number of infections with DENV (139). Vector control methods include various strategies: environmental, biological, chemical, cultural, and integrated pest management. Because the *Ae. aegypti* mosquito is a container breeder, removal of any potential breeding containers is important to reduce the vector population (103). The application of insecticides and personal protective measures, such as the use of repellents or wearing of long-sleeved shirt and long pants, are also advised (103). The development of *Wolbachia*-infected mosquitoes has opened up a new avenue for biological mosquito control (86). *Wolbachia* infection has been shown to be stable in both laboratory and field settings (59; 86). The mosquito strain that carries the bacterium has also been shown to be less fit and more resistant to DENV infection in the laboratory (11; 87; 136). Moreover, the *Wolbachia* increased the length of the extrinsic incubation period that could further reduce the transmission of DENV (144).

DEET

One of the most widely used personal protective measures against arthropod-borne diseases is the application of insect repellent to exposed skin or clothing. *N, N*-diethyl-*m*-toluamide (DEET) was first synthesized by the United States Department of Agriculture using funding from the Department of Defense (30). DEET was originally used in the military settings. Starting in 1957 it became available for general public use and it is currently the most widely used insect repellent compound in the world (24; 137). DEET has been demonstrated to be very effective and to have very good safety features (6; 24; 66; 82). It is widely available with varying concentrations that range from 5-100%. The concentration corresponds to the length of protection it can provide against mosquitoes. However, the efficacy of DEET plateaus at a concentration of 50% (20). The length of protection also depends on the formulation of the compound. The current formulation of DEET is a long-lasting emulsion in which 30% DEET can actually lasts longer than 70% percent DEET with the old formulation. There are several reports of DEET-related medical cases, but these were typically caused by the patient's underlying medical conditions or excessive/incorrect applications of DEET (24; 137).

DEET has been demonstrated to have three activities against mosquitoes. It can act as toxicant, irritant, and repellent. In general, chemicals are considered toxicants when they can kill or cause knock down in arthropods. Irritants and repellents both cause arthropods to avoid areas where the chemicals are applied. However, the two activities are fundamentally different. For an irritant to work, it requires the arthropod to make a direct physical contact with the applied surface. Conversely, a repellent does not require direct physical contact.

There have been reports of DEET insensitivity in insects including mosquitoes (106). The insensitivity can stem from the genetic makeup of the mosquitoes or from their habituation (122; 123; 135). A study by Stanczyk showed that pre-exposure to DEET rendered the mosquitoes less sensitive to DEET in subsequent exposure (122). This information is important because DEET may not give the length of protection as has been previously believed. Moreover, it also raises the question if prior exposure to DEET can alter mosquitoes' subsequent behaviors.

Mechanism of Action

Although DEET has been available to the public for decades, there is still no consensus on its mechanism of action. Three different hypotheses have been proposed. Ditzen et al. reported that DEET directly inhibits the OR83B of *Anopheles gambiae*, thus masking the attractant compound 1-octen-3-ol (32). In the second hypothesis, Pellegrino et al. suggested that DEET acts as a confusing agent that makes the insect unable to process the odorant information thus explaining its effectiveness on a wide range of insects (95). Thirdly, Syed and Leal reported that a specific OR of *Culex quinquefasciatus* could actually recognize DEET molecules and consequently actively avoid its source (125). Because DEET has a fixative effect that can reduce the volatility of attractant chemicals, experiments has to be designed carefully to avoid confusing this fixative effect with the true irritancy or repellency effect of DEET (104).

Multiple studies have tried to elucidate specific mechanism and receptors that cause the toxicity, irritancy, and repellency response in insects. The toxicant action of DEET was suggested to be from its activity as a cholinesterase inhibitor in both insect

and mammal (28). However, there is still not enough information showing how DEET elicits irritancy or repellency responses.

Knowledge of how DEET elicits irritancy and repellency responses is important in aiding future development of new chemicals. A study using orco mutants of the *Ae. aegypti* mosquito found that the mutants displayed repellency but not irritancy. A study by Kain et al. (64) found that the IR40a in *Drosophila melanogaster* played a role in DEET perception. The fact that the IR is more conserved across the insect world and that DEET can repel most insects further suggests that it might play a bigger role than OR in DEET perception (104). These studies suggest that different receptors might play different roles in eliciting what initially seems to be similar avoidance behavior to DEET.

INSECT BEHAVIOR ALTERATION

Alteration of insect behavior can be harnessed to human advantages. Indeed development of some traps and repellents follow the principle of manipulating insect behavior using chemicals. Chemical exposure may cause insects to become attracted, irritated, or repelled. Infection by pathogens may also alter or cause damage to specific infected organs of the insect. This may in turn lead to behavioral changes (60). It is important to study potential behavioral changes in vectors since this can provide valuable information to help combat spread of diseases. The behavior alteration is usually studied in the form of increased biting or prolonged blood-feeding; these two behaviors will increase the transmission rate of the parasites into their vertebrate host. Thus, they are epidemiologically important (71; 115). The blood-feeding behavior change is detrimental to the vertebrate hosts because of increased pathogen transmission level and is also damaging to the vector because of increased blood-feeding rates. The longer duration

exposes the vector to a higher risk of being killed by the vertebrate host via interruption of the blood-feeding process (71).

Parasitic Infection

Various parasites have been demonstrated to cause behavioral changes in their vector's behaviors. Moreover, in some parasites the vector behavioral changes depend on the parasite life cycle stage. For example, *Leishmania* infection can increase the probing rate of the sand fly vector by producing a gel plug in the anterior midgut of the sand fly. This plug is only produced by the infective promastigote form of *Leishmania* (105; 111; 112). Several other studies using malaria parasites gave an even more complete picture of vector behavior manipulation by parasites. The oocyst stage is not infectious to the vertebrate hosts and it is advantageous to parasite survival if the vector does not risk itself by refeeding (4; 115). Once the oocysts mature and produce infective sporozoites, these need to be transmitted to a vertebrate host in order to complete the life cycle. Therefore, once again the vector seeks a blood meal. Indeed, vector behavioral manipulation that fits the parasite life stage have been observed in the laboratory. Infection of *Ae. aegypti* by *Plasmodium gallinaceum* decreases the vector biting rate during the oocyst stage. However, the biting increases when the sporozoites emerge (70). Another study with *P. yoelii nigeriensis* and *An. stephensi* mosquitoes also yielded similar results (5). Field studies also found that human malarial parasites also increase the biting rates and the blood meal size of the infected anopheline mosquitoes (69; 71). Unfortunately, the exact mechanism of how this behavioral change is exerted is still unknown. The reduction of apyrase in infected mosquitoes has been proposed as a plausible explanation. Apyrase is an enzyme in mosquito saliva that enables the blood-feeding process by disrupting the

host blood clotting mechanism; therefore, the reduction of apyrase increases the probing/biting rate (21; 108) by reducing the ability of the vector to locate blood. Another theory is that infection alters the vector satiety receptor causing them to ingest more blood (71). Interestingly, Cator *et al*. (18) demonstrated that heat-killed *Escherichia coli* could also induce the same behavioral change. This is a concern about whether the behavioral change is a result of general vector immune response or active parasitic manipulation. Interestingly, stage-specific human attractiveness has also been documented. Humans with transmissible gametocytes were more attractive to the mosquitoes than those who were uninfected or infected with the asexual/nontransmissible stage of parasite (74).

Arboviral Infection

Vector behavioral alteration has also been observed in the arbovirus-infected population. *Aedes triseriatus* mosquitoes with La Crosse virus infection show a reduced blood engorgement level (45; 61). Mosquitoes are more likely to refeed after a partial blood meal, while those that have taken near or complete blood meal will find a place tor rest to digest the blood and start producing eggs (Figure 7) (45; 61; 96). Therefore, a reduced blood engorgement level may cause an increase in the vectorial capacity. A similar result was also reported in *Cx. pipiens* with disseminated Rift Valley fever virus (131). The DENV has also been reported to alter *Ae. aegypti* behaviors in laboratory settings. The mosquitoes are reported to be less likely to initiate feeding when infected with DENV-2 and take longer to complete a blood meal; thus, they are more prone to obtain an incomplete blood meal and are more likely to refeed (84). Another study showed increased locomotor activity in DENV-infected mosquito compared to controls.

When combined with the vector blood-feeding behavioral change, this could potentially increase the vectorial capacity (81; 83). Moreover, just the probing action itself without any blood intake can transmit the pathogen; thus the vectorial capacity increase may be even higher.

Qualls et al. have conducted studies using Sindbis virus (SINV)-infected *Ae. aegypti* mosquitoes and found that the infection renders the mosquitoes less sensitive to DEET (100-102). It is speculated that because the brain is a site of heavy viral replication during arbovirus infection in mosquitoes, it is likely that the damage from the replication causes this behavioral alteration. In addition, the infected mosquitoes display more aggressive feeding behavior compared to uninfected controls (101; 102).

Dengue Infection in Mosquito

Not all mosquitoes can be infected by DENV. The *Aedes spp*. mosquitoes from the subgenus *Stegomyia* are susceptible to infection and some are competent to transmit the disease. The vector competence is determined by several factors: the mosquito susceptibility to infection, the mosquito ability to become infectious, and the propensity to bite the appropriate mammalian host (17). The *Ae. aegypti* mosquito is the primary vector for DENV transmission. The female mosquito can acquire the virus from an infectious blood meal. The virus then infects and replicates in mosquito midgut epithelial cells, from where it subsequently spreads to other parts of the body. Eventually, the virus will reach the salivary gland. If the virus can infect the salivary gland then be secreted in the saliva, the mosquito becomes infective and capable of transmitting the virus during its next blood meal (40). Salazar et al. (29) conducted a kinetic study using *Ae. aegypti* mosquitoes orally infected with DENV-2. They found mosquito midgut and abdomen to

be infected as early as 2 days post infection (dpi), with 76-95% of mosquitoes becoming infected by 7 dpi. However, these organs displayed reduced viral replication after 11 and 18 dpi, respectively. That study also showed that head tissues became infected 4 dpi, respectively. This organ continued to be heavily infected until the last observation 21 dpi (Figure 8). The time from when a mosquito ingests an infectious blood meal to the time it becomes infective is called the extrinsic incubation period. The length of the extrinsic incubation period is determined by the dose of virus ingested, the temperature in the environment, the virus strain, and the mosquito species. Lower viral dose and lower temperature warrant longer extrinsic incubation period and higher viral dose and higher environmental temperature expedite the incubation process (17).

Several nonmosquito factors help determine the infection process in the mosquito (17; 56). A study by Nguyet et al. (90) showed that, depending on the virus serotype, the infectious virus dose needed to infect 50% of mosquito tested (ID50) for DENV is between 6.3 $\log_{10}$ to 7.5 $\log_{10}$ viral RNA copy/mL of plasma of infected human. The human host was shown to be infective from around 1.5 days before the onset of symptoms to a few days after the febrile episode has subsided. Highly viremic patients were the more infectious blood sources (90). Because most DENV infections are asymptomatic (9; 42), it is very likely that these subsets of infected people also contribute to greater transmission of DENV in the population due to their mobility (17).

Artificial Infection in Mosquito

For research purposes, mosquitoes have been artificially infected in laboratory settings. Mosquitoes can be infected by allowing them to feed on a blood source that has been spiked with virus. The blood is usually contained in a sausage casing or feeding

glass bell with a membrane surface for the mosquito to probe and feed from. This method is easy and can expose hundreds of mosquito to virus at once. However, the infection rate is rarely uniform and it is very difficult to ascertain which mosquitoes have the disseminated infection (46). The other method is called intrathoracic inoculation. By using a fine glass needle syringe, the side of the mosquito thorax region is punctured to introduce the virus solution. This method is very labor intensive and time-consuming, but has a very high infection rate and can create a uniform test population for assays (47).

Chemical Pre-exposure

Insects are not always uniformly exposed to chemicals in nature. Interestingly, multiple studies have shown that chemical pre-exposure or priming can alter the subsequent behavior of insects (117). The altered behavior can be displayed in the form of changes in locomotor activity, blood-feeding behavior, or even reaction to the same or different chemicals. For example, the locomotor behavior of *Blatella germanica* was lower when it was pre-exposed to DEET. In contrast, *Rhodnius prolixus* was more active after pre-exposure to DEET. Stanczyk et al. (122) reported decreased repellency to DEET in *Ae. aegypti* after the mosquitoes had undergone a pre-exposure process to the chemical. Another study by Thany et al. (128) demonstrated that imidacloprid pre-exposure was able to reduce the repellency of *Ae. aegypti* to lemon oil and DEET.

The exact mechanisms of these behavior alterations are still not clear. However, because the brain controls behavior and most chemicals tested in these behavioral studies are agents that affect the nervous system of insects, it is very likely that the nervous damage or habituation through olfactory saturation plays a role in these changes (117; 128). A study by Vinauger et al. (135) also suggested that this behavioral change could

just be a virtue of the insect learning ability to associate stimulus and reward; this behavioral change can be interrupted by memory damaging procedures such as cold shock or cycloheximide.

HIGH THROUGHPUT SCREENING SYSTEM

The high throughput screening system (HITSS) chambers were originally developed as a quick screening method to find new chemicals that have the capability to kill, or induce irritancy, or spatial repellency in mosquitoes. The system consists of different components, and depending on how the chambers are configured, the system can be used to screen chemicals for toxicity, irritancy, or repellency against insects.

There are two main components of the HITSS chambers, a metal chamber and a Plexiglas® chamber. The metal chamber has an inner cylinder that can be lined with fabric material. This fabric material can be treated with the chemical of choice or with solvent as a control. For the irritancy assay, one metal chamber is attached to one Plexiglas® chamber (Figure 9). The mosquitoes will then be released into the metal chamber. If the chemical irritates the mosquitoes, they will attempt to fly or escape to the Plexiglas® chamber through a butterfly gate that is opened for a certain period of time. For the spatial repellency assay, a Plexiglas® chamber is connected at each end with two metal chambers, one metal chamber contains a chemically treated fabric and the other one contains solvent treated fabric. The mosquitoes are released into the Plexiglas® chamber and if the chemical has a repellency effect, the mosquitoes will fly or be repelled into the chamber with solvent only. Likewise, if the chemical is an attractant, the mosquitoes will be more likely to fly or be attracted into the chamber with the chemical.

The mosquitoes can be collected from either assay to assess the 24 hours toxicity effect of the chemical (44).

The escape rate is calculated by correcting the total number of chemical-irritated mosquitoes with the number of mosquitoes that are knocked down and the number of mosquitoes that fly to the Plexiglas® chambers as a result of random movement. Below is the calculation.

$$\mathrm{NKD} = \mathrm{NT} - \mathrm{TMKDMET}$$

NT= Number of mosquitoes in treated device

TMKDMET= Number of knock down mosquitoes in the metal chamber of treated device

$$\mathrm{C2PE} = \left(\frac{(\mathrm{NC} - \mathrm{CCNT}) - (\mathrm{NKD} - \mathrm{TCNT})}{\mathrm{NC} - \mathrm{CCNT}}\right) * 100$$

C2PE= Percentage of mosquito escaping corrected for control escaping and mortality

NC= Number of mosquitoes in control device

CCNT= Number of mosquitoes escaping in control device

TCNT= Number of mosquitoes escaping in treated device

AIMS OF THE STUDY

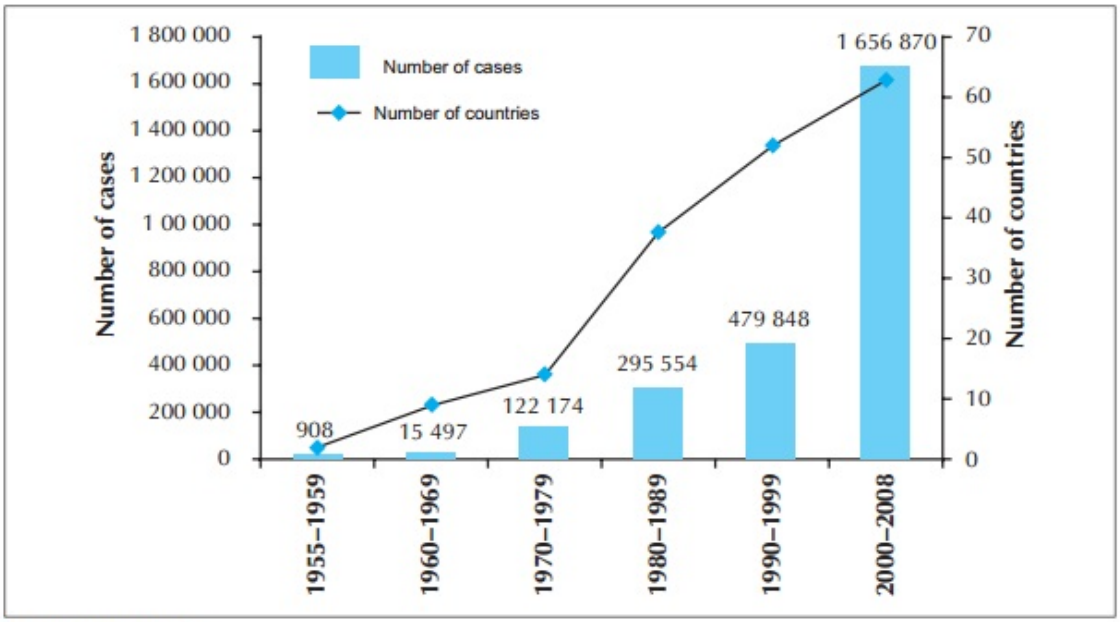
The aim of this study was to assess potential behavioral changes in *Ae. aegypti* that may result from arbovirus infection or chemical pre-exposure. Our study focused on two settings that we considered to have significant epidemiological impacts: 1) when the mosquito is infected with DENV, and 2) after the mosquito is pre-exposed to DEET.

To Determine If the Behavioral Response to DEET is Altered in Dengue Virus-infected *Aedes aegypti* Mosquitoes.

As noted above, infections in mosquitoes have been demonstrated to alter various aspects of mosquito behaviors: blood-feeding, movement, and response to chemicals. Here we sought to elucidate whether DENV-1 infection in female *Ae. aegypti* mosquitoes would alter their irritancy behavioral response towards DEET in the HITSS chamber assay.

To Determine If Prior Exposure to DEET Can Alter the Subsequent Blood-feeding Behavior of *Aedes aegypti* Mosquitoes.

Prior exposure to chemicals can alter subsequent behavior of some insects. Unfortunately, in the real life setting, DEET is not applied uniformly in the population. This is due to issues such as cost, greasy feeling, unappealing odor, feeling that it is a hassle, or plasticizer capability (25; 104). We were interested to see if prior exposure to DEET would alter any aspect of the subsequent blood-feeding behaviors (landing, probing, or blood engorgement level) in *Ae. aegypti* mosquitoes; alterations could have important implications in vectorial capacity.



Source: www.who.int.

Figure 1. Number of global reported DENV cases.
Figure adapted from WHO (139).

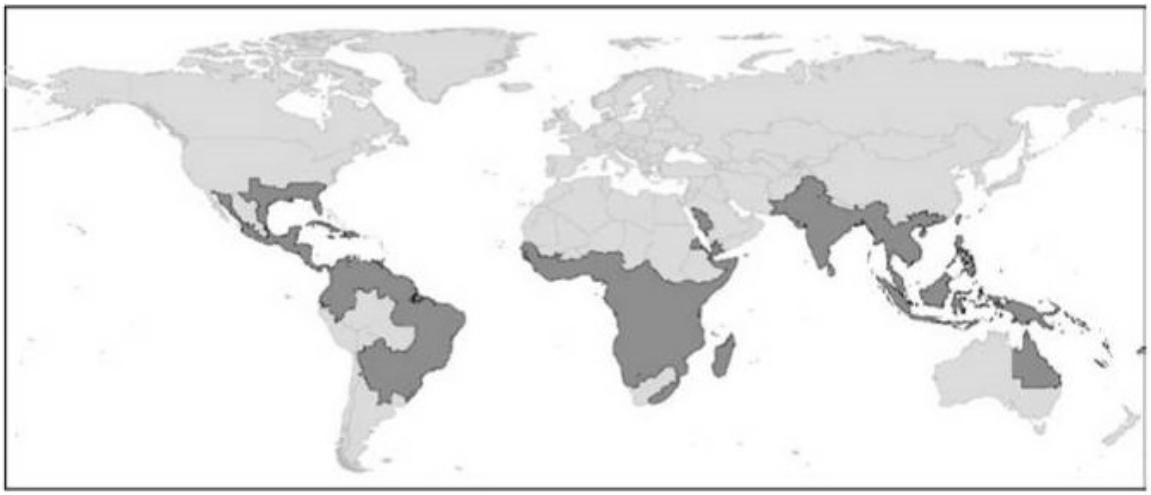


Figure 2. The global distribution of *Ae. aegypti* mosquitoes provided in a state/country scale. Areas in dark grey color indicate places where *Ae. aegypti* has been reported to live. Figure adapted from Rogers et al. (110).

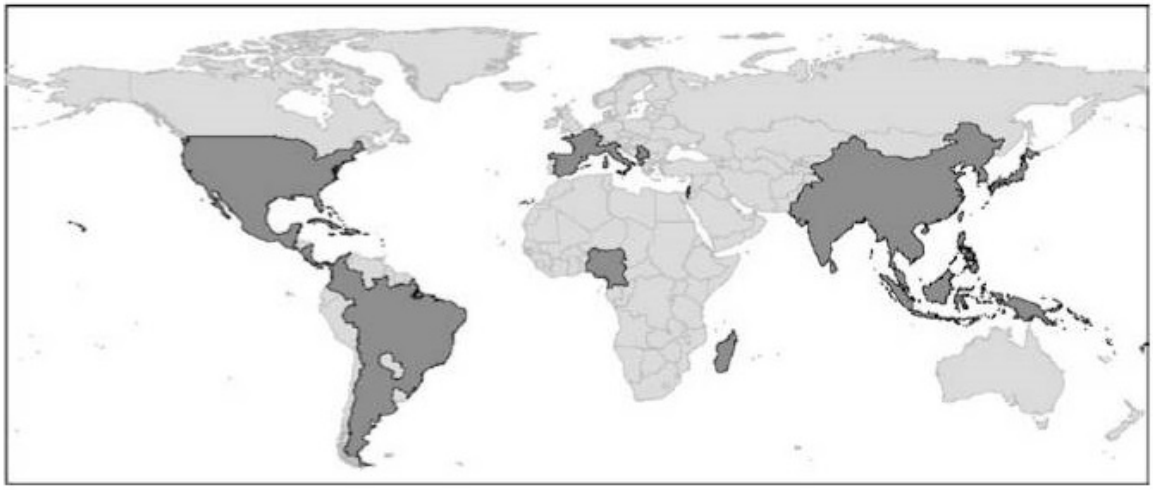


Figure 3. The global distribution of *Ae. albopictus* mosquitoes provided in a state/country scale. Areas in dark grey color indicate places where *Ae. albopictus* has been reported to live.

Figure adapted from Rogers et al. (110).

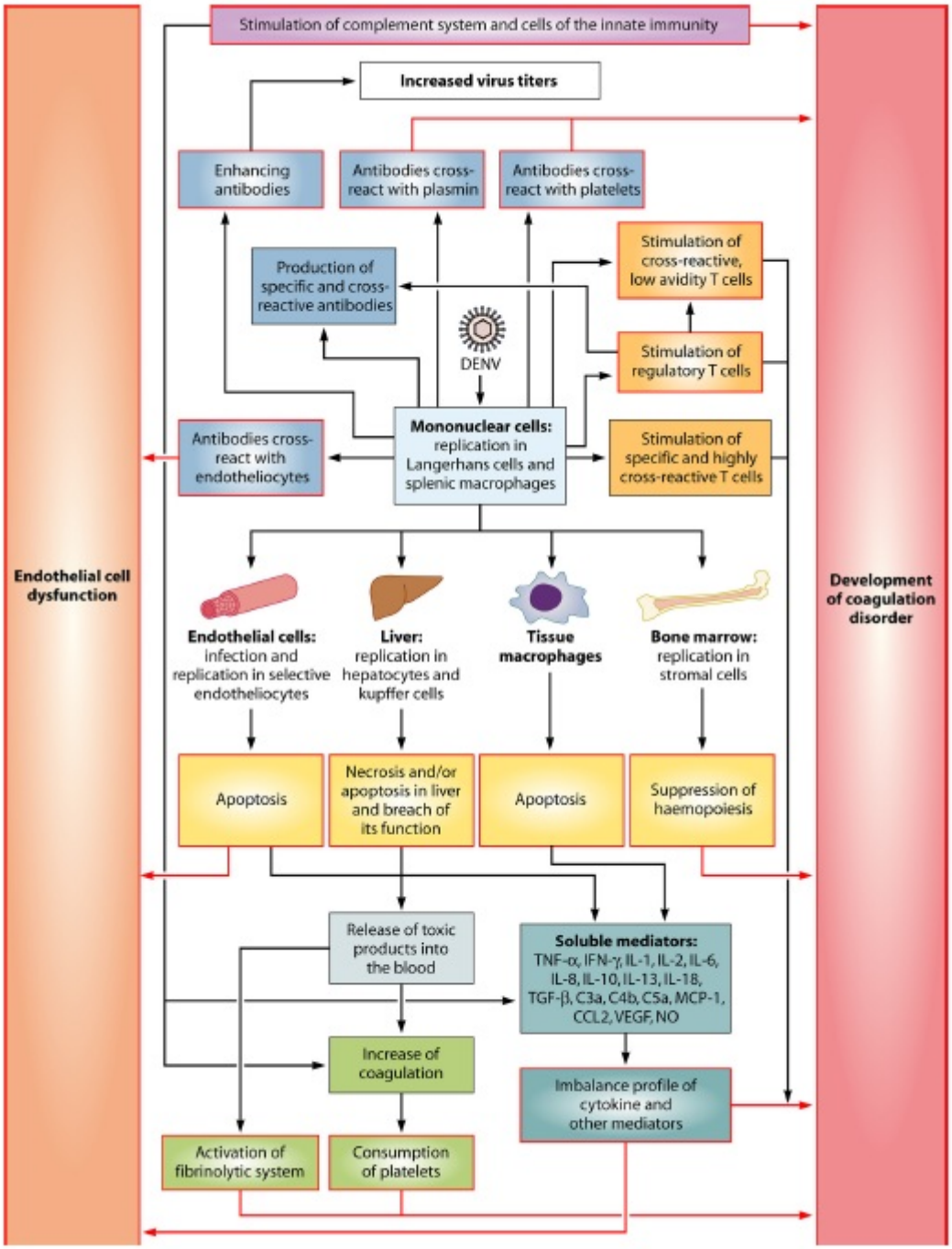


Figure 4. Dengue pathogenesis in the human host.

This diagram shows how DENV infection can cause immunopathogenesis which subsequently leads to the vascular leakage that is the hallmark of dengue hemorrhagic fever/dengue shock syndrome. Figure adapted from Martina et al. (85).

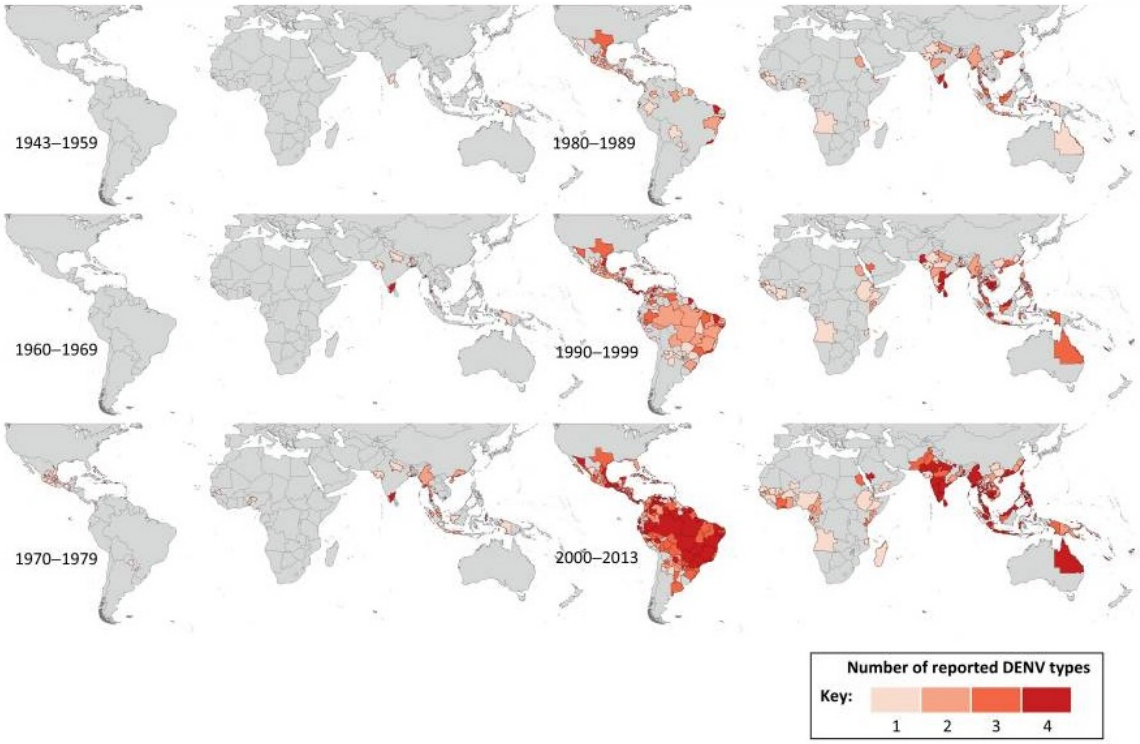


Figure 5. The global distribution of DENV from 1943 to 2013 by serotype. Figure adapted from Messina et al. (88).

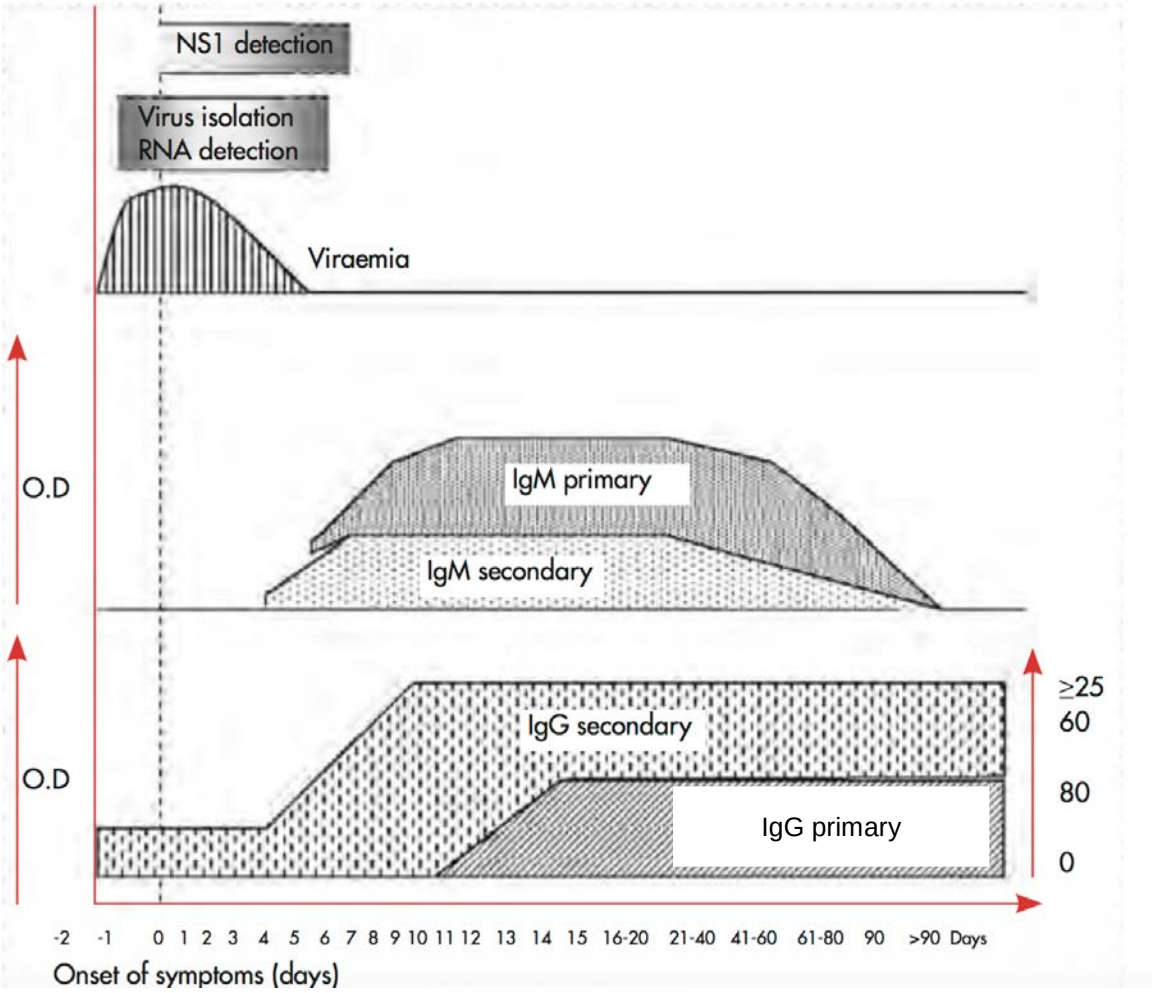


Figure 6. The timeline of DENV infection and the corresponding diagnostic methods used within those timeframes.

Figure adapted from WHO (139).

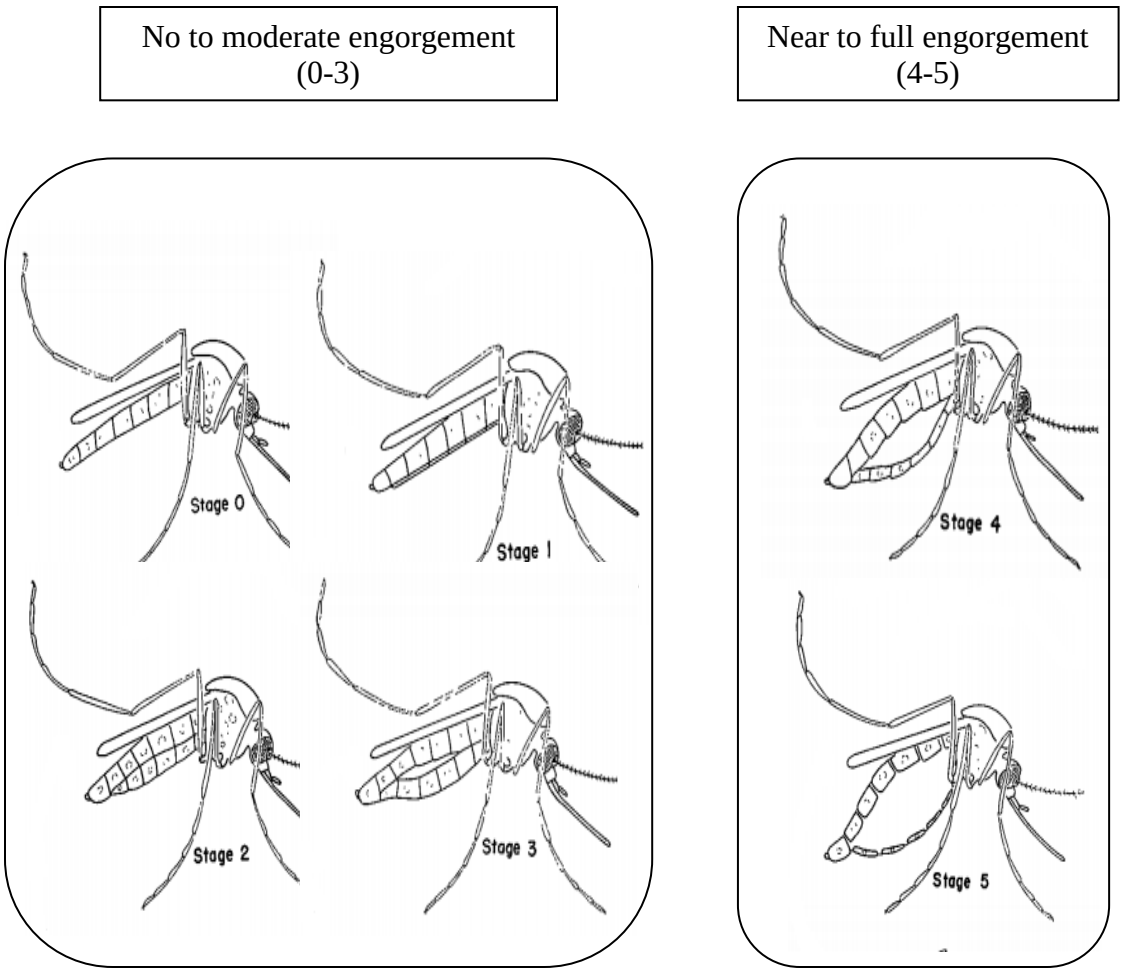


Figure 7. Blood engorgement level stages in *Ae. aegypti* mosquitoes.
Stage 0-3 are categorized as no to moderate engorgement or partial blood-feeding. Stage 4-5 are categorized as near to full engorgement.
Figure adapted from Pilitt and Jones (96).

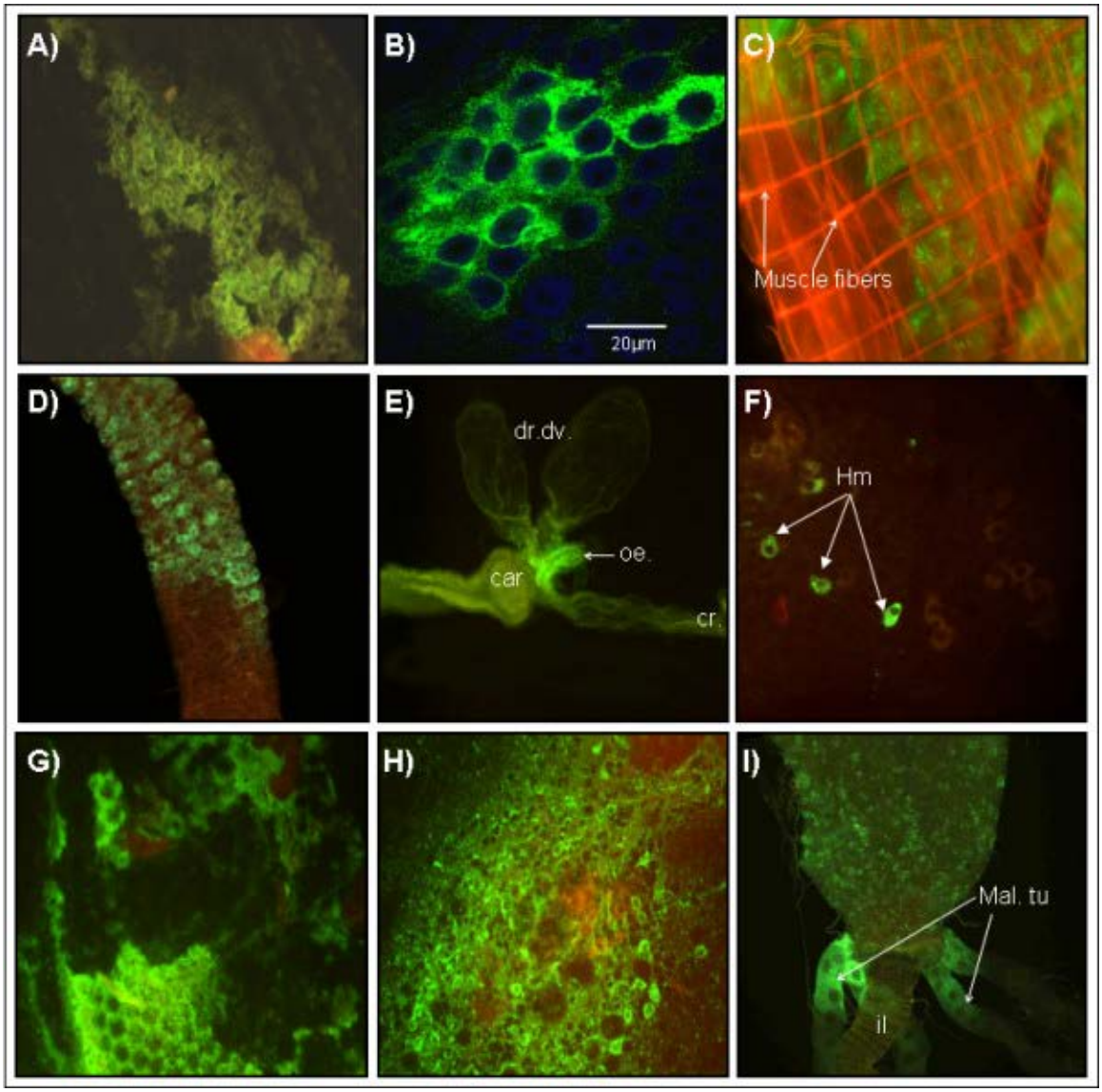


Figure 8. Various organs of *Ae. aegypti* mosquito during DENV infection with green color indicating the presence of DENV in the organ.
(A) Fat body, (B) midgut epithelial cells, (C) the muscles surrounding midgut epithelial cells, (D) anterior midgut, (E) esophagus, (F) hemocytes, (G) ommatidia, (H) brain, (I) malphigian tubules.
Figure adapted from Salazar et al. (44).

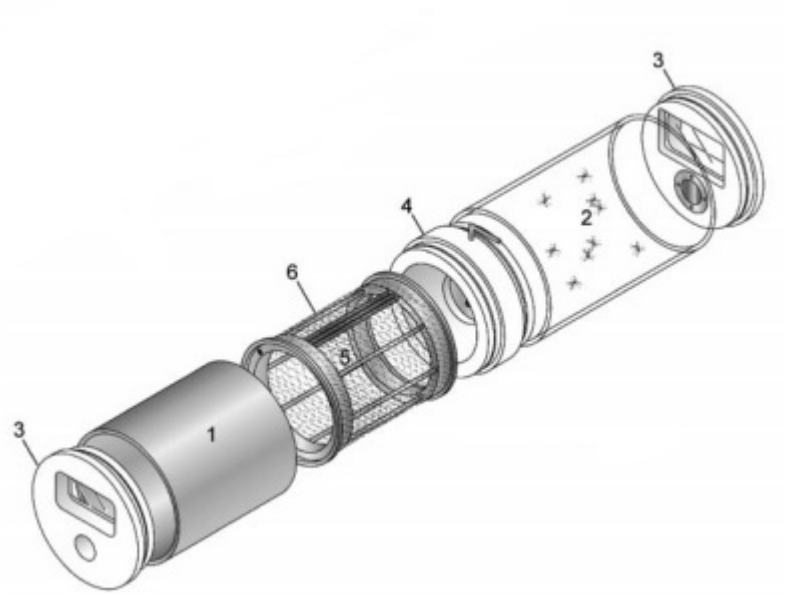


Figure 9. HITTS chamber in the contact irritancy assay configuration.
The components are: 1. Treatment metal; 2. Clear Plexiglas; 3. End cap; 4. Linking cap with butterfly gate; 5. Treatment drum; 6. Treatment net. Figure adapted from Grieco et al. (44).

CHAPTER 2: Exploring the Effect of Dengue Virus Infection on the Response of *Aedes aegypti* to DEET

Submitted as: Sugiharto VA, Murphy JR, Turell MJ, Olsen CH, Stewart VA, Colacicco-Mayhugh MG, Grieco JP, Achee NL to the Journal of Medical Entomology (submission number: JME-2016-0019).

Sugiharto VA designed and conducted the experiments, analyzed the data, and wrote the manuscript.

Murphy JR analyzed the data and edited the manuscript.

Turell MJ designed the experiments, analyzed the data, and edited the manuscript.

Olsen CH designed the experiments, analyzed the data, and edited the manuscript.

Stewart VA analyzed the data and edited the manuscript.

Colacicco-Mayhugh MG designed the experiments and edited the manuscript.

Grieco JP designed the experiments, analyzed the data, and edited the manuscript.

Achee NL designed the experiments, analyzed the data, and edited the manuscript.

ABSTRACT

No licensed vaccine or antiviral drug against DENV is available; therefore, most of the effort to prevent this disease is focused on reducing vector-host interactions. One of the most widely accepted methods of blocking vector-human contact is to use insect repellents to interfere with mosquito host-seeking behavior. Some arboviruses can replicate in the nervous system of the vector, raising the concern that arboviral infection may alter the insect behavioral response toward chemical stimuli. Three different *Aedes*

aegypti (L.) mosquito cohorts: DENV-1-injected, diluent-injected, and uninjected were subjected to behavioral tests using a high throughput screening system with 2.5% DEET and 0.14% DEET on 1, 4, 7, 10, 14, and 17 days post-injection (dpi). All test cohorts exhibited significant contact irritancy or escape responses when they were exposed to 2.5% or 0.14% DEET. There were no behavioral changes among the test populations when they were exposed to 2.5% DEET. However, we found no biologically relevant irritancy response change in DENV-1 infected *Ae. aegypti* mosquitoes when they were exposed to DEET. Further studies evaluating the effects of other arboviral infections on insect repellents activity are necessary in order to provide better recommendations on the prevention of vector-borne disease transmission.

Keywords: *Aedes aegypti*, DEET, behavior, dengue

INTRODUCTION

Mosquito behaviors: such as host-seeking, landing, probing and biting, are driven by chemical signals in the environment that are recognized by the mosquito olfactory system (55). The olfaction process starts when the odorant molecule enters pores on sensilla and becomes solubilized by binding to the odorant binding proteins. Through pH changes, eventually the molecule is released and picked up by the odorant receptors on the dendrite of the olfactory receptor neurons. These neurons relay the message to the corresponding glomerulus in the antennal lobe of the brain. The information is then continued to the mushroom body, where learning and memory function reside, and the lateral horn, where it directs the appropriate behavior response to the odorant stimulus (48; 124; 132).

Dengue virus is considered the most important arbovirus in the world by the WHO (139). A wide range of manifestations can be seen with DENV infections, including asymptomatic infection, mild undifferentiated fever, dengue fever with the hallmark muscle and joint pain, and the deadly dengue hemorrhagic fever/dengue shock syndrome (139). Approximately 2.5 billion people are at risk for infection with 100 million infections annually. Out of these 250,000- 500,000 are severe cases (139; 140). The main vector for DENV infection is the cosmopolitan *Ae. aegypti* (L.) mosquito, which thrives in the areas between 40^{o}N and 40^{o}S latitude.

The lack of a DENV vaccine and antiviral drugs has resulted in a reliance on vector control interventions as the primary method of dengue disease prevention. These typically focus on reducing human-vector contact (139; 140). Topical repellents work by either masking host odor or eliciting aversion responses that sequentially inhibit mosquitoes from obtaining a blood meal (32; 125). Repellents are frequently used to prevent mosquito bites and represent one potential tool for personal protection against DENV infection. The most widely used topical insect repellent is N, N-Diethyl-meta–toluamide, more commonly known as DEET (137). DEET has a good safety profile and has been proven to be very effective in protecting against insect bites (37; 92). Interestingly, the scientific consensus on the mechanism of action still remains elusive. Currently, three different proposed theories for DEET mechanism exist: 1) as a masking agent, 2) as a true repellent that triggers repulsion, and 3) as a confusant (12; 31; 33; 125).

The influence of pathogen infection on numerous vector species' behaviors has been reported in various publications. *An. gambiae* mosquitoes infected with the sporozoite stage of *P. falciparum* are more attracted to human odors and feed more

frequently with larger blood intake as compared to uninfected counterparts (71; 121). Arboviral infections in mosquitoes have also been shown to elicit behavioral changes. Reduced blood intake has been reported in *Ae. triseriatus* mosquitoes infected with La Crosse virus, which causes them to refeed more frequently when compared to the noninfected population (45; 61). In another study, *Cx. pipiens* with a disseminated Rift Valley fever virus infection were less able to obtain a blood meal than were sibling mosquitoes without a disseminated infection (131). Thus, the mosquitoes with a disseminated infection might attempt feeding on numerous hosts and be able to transmit numerous times during a single ovarian cycle. *Aedes aegypti* mosquitoes infected with SINV require significantly less time to start feeding on a DEET treated membrane than uninfected, matched *Ae. aegypti*. However, the SINV-infected mosquitoes took a longer time to complete their feeding than the uninfected controls (102). A similar alteration in blood-feeding behavior was reported in *Ae. aegypti* mosquitoes infected with DENV; the mosquitoes started feeding sooner but required more time to finish (97). Another study with DENV-infected mosquitoes demonstrated increased movement or locomotor activity when measured and recorded using the *Drosophila* activity monitoring machine. However, no study has yet investigated the effect of DENV-infection on *Ae. aegypti* mosquito response to DEET (81).

Previous studies suggested that there might be an association between the infection of the mosquito nervous system of the vector and these behavioral changes (38; 97). Moreover, arboviruses, such as DENV, SINV, and West Nile virus, often replicate and remain at elevated levels within the mosquito's nervous system during infection (13; 41; 114). This pathophysiology may interrupt behavioral responses to chemicals,

potentially reducing the efficacy of interventions. Because previous studies have shown that *Ae. aegypti* mosquitoes with disseminated SINV exhibit decreased sensitivity to DEET (100-102), it is important to investigate if a similar behavioral change also occurs in DENV-infected mosquitoes.

The objective of this study was to determine the effect of DENV-1 infection on the contact irritant (escape) response of *Ae. aegypti* when exposed to two concentrations of DEET. Responses were measured on different days post-DENV injection (dpi) to correlate with expected viral RNA copy number within the mosquito nervous system (114).

MATERIALS AND METHODS

Mosquito Populations.

Aedes aegypti (Liverpool strain) were colonized at the Walter Reed Army Institute of Research (WRAIR) following standard rearing protocols. Eggs were hatched and larvae fed with Cichlid Gold TM fish food (Hikari USA, Hayward, CA). After molting into the second instar larvae, they were separated into groups of 50 and maintained at 28^{o}C, 80% RH and 12 hours light-dark cycle. After the larvae pupated, the male and female pupae were sorted using a pupal separator and then estimated into groups of approximately 250 females using a pupal estimator tool. They were then put into 3.9-litre bucket rearing cages (white plastic round bucket with mesh covered plastic caps). After eclosion, adults were provided with cotton balls soaked in 10% sugar solution in water as a carbohydrate food source. All adult mosquitoes used in testing were 3-5 days old females. Three cohorts were used: 1) DENV-1 injected; 2) diluent-injected

(Minimum Essential Medium with Earle's salts and L-glutamine [Corning Life Sciences, Manassas, VA]); and 3) uninjected (control).

Intrathoracic Injections.

Mosquitoes were cold-anesthetized at -20^{o}C for 120 seconds then transferred to a chill table. Using a fine glass syringe, 0.3 µl of solution (DENV-1 or minimum essential media as diluent) was injected into individual mosquito thoraces (113). The DENV-1 used was the D02-005 strain isolated from human in Thailand in 2002. The uninjected cohorts were cold-anesthetized only. All populations were transferred to holding cups and maintained at 28^{o}C and 80% RH with access to 10% sugar solution without being starved until the specific day of testing, which were 1, 4, 7, 10, and 14 dpi. An additional observation day (17 dpi) was added to increase the ability to detect any difference in behavioral response in the 0.14% DEET experiments.

Behavioral Assay

Assay Device

The high throughput screening system (HITSS) behavioral assay was used to measure the response of mosquitoes to DEET (Grieco et al. 2005). The system is composed of a cylindrical metal chamber that can be joined together with a Plexiglas® cylinder chamber using a plastic cap affixed with a butterfly valve. The treated substrate was attached on the inside of the metal chamber using magnets. A clear Plexiglas® escape chamber is attached to the assay to quantify the numbers of mosquitoes that escape (displayed contact irritancy) as compared to a matched control. Both the metal and Plexiglas chambers are equipped with a small portal covered with a dental dam through

which mosquitoes can be introduced and removed, prior to and after the assay, respectively.

Test Material Treatment

Neat grade DEET mixed to a 5% solution in ethanol was provided by the United States Department of Agriculture (USDA), Beltsville, MD. Initial experiments were conducted using 2.5% DEET. For the second set of experiments, a dose response evaluation was conducted to determine optimal DEET concentration to be tested against infected mosquitoes. The lowest dose of DEET that resulted in a significant irritant response evaluated in this experiment was 0.14%. We applied 1.3 ml of either absolute ethanol (Sigma-Aldrich, St. Louis, MO) as a control or the appropriate concentration of DEET in ethanol uniformly on a sheet of polyester organdy netting (330 cm^2) (G Street Fabric, Rockville, MD). The treated nettings were allowed to air dry for 15 minutes prior to being inserted into the chambers for testing.

Contact Irritancy Assay (CIA)

Ten female *Ae. aegypti* mosquitoes from each test cohort were introduced into the metal chambers of separate assays containing organdy netting treated with either DEET or ethanol-only (matched control). Mosquitoes were acclimated for 30 seconds after which time the butterfly valves were opened for a 10 minutes test period to allow mosquitoes to escape into the attached Plexiglas chamber. Plexiglas chambers were covered with felt material in order to control for behavioral activity influenced by the presence of light. The number of mosquitoes within each chamber was recorded and classified as alive or knocked-down (dead or alive but unable to move normally or not moving, this number was used to calculate the mortality-corrected percent escape).

Behavioral assays were conducted on various dpi to allow for the dissemination of the virus in the mosquito. All mosquitoes were transferred from the assay chambers to individual holding cups according to which chambers the mosquitoes were collected from and then freeze-killed at -20^{o}C for 30 minutes. Heads were removed from bodies and stored at -80^{o}C in preparation for RNA extraction to monitor DENV-1 infection. A minimum of eleven replicates were performed for each test cohort for each test dpi.

Molecular Assay

RNA Extraction

Thirty mosquito heads from each day post-injection were chosen randomly. Total RNA from mosquito heads were extracted using the Exiqon miRCURY RNA Isolation Kit - Cell and Plant (Exiqon Inc., Woburn, MA). The purified mosquito RNA was stored at -80^{o}C.

Reverse Transcriptase Real-Time PCR

The amount of DENV-1 viral RNA was quantified using a method previously described (50). DENV-1 armored RNA from Asuragen (Asuragen Inc., Austin, TX) was used as a standard.

Data Analysis

Contact irritancy was measured by correcting the number of mosquitoes that escaped the DEET chamber with the number of mosquitoes that escaped the corresponding control/solvent chamber. Prior to performing this correction, however, both the treatment and control escapees were adjusted to take into account the number of knocked-down mosquitoes in either chamber. The number was reported as mortality-

corrected percent escape. To determine the level of irritancy within test cohorts, a Wilcoxon rank sum test was performed to examine the difference in the number of mosquitoes that had escaped the DEET-treated chamber as compared to the control chamber. The difference in mortality adjusted average percent escape and the $\log_{10}$ viral RNA were compared among groups and days post-injection using a two-way ANOVA followed by a Tukey's post-hoc test ($P < 0.05$) using SPSS 22.0 software (IBM Corp., Armonk, NY). The analyses above were performed using SAS v. 9.3 software (SAS Institute, Inc., Cary, NC).

RESULTS

Contact Irritancy (Escape) Response Against 2.5% DEET

When exposed to 2.5% DEET in the HITSS chambers, all *Ae. aegypti* test populations displayed significant contact irritancy compared to corresponding matched HITSS control chambers with ethanol-treated lining ($P < 0.01$) (data not shown). This was consistent for all dpi. However, there was no significant difference in the rate of escape among the groups in any of the dpi test populations (Figure 10). Because of the high rate of mortality, the high dose of DEET that was utilized might have masked true behavioral difference caused by the DENV-1 infection. Therefore, we set up dose-response experiments to further refine the DEET concentration that could still elicit escape. We found 0.14% was the minimum concentration of DEET that could still induce an escape response in the uninjected *Ae. aegypti* cohort (data not shown).

In behavioral trials conducted with 2.5% DEET, the viral loads on each day post-injection were not measured; instead, random samples of DENV-1 injected mosquitoes, diluent-injected mosquitoes, and control mosquitoes from various dpi were taken and

analyzed for DENV-1 RNA. All DENV-1 injected mosquitoes had detectable DENV-1 RNA, while the other test groups did not (data not shown).

Contact Irritancy (Escape) Response Against 0.14% DEET

As previously observed at a dose of 2.5% DEET and throughout the dose-response experiments, all *Ae. aegypti* test populations displayed significant contact irritancy against 0.14% DEET compared to their respective control group ($P < 0.01$) (data not shown). There was no significant difference in escape among the three test cohorts on 1, 4, 7, 14, and 17 dpi. However, significantly higher escape rates were seen on 10 dpi ($F = 5.39$; df = 2,412; $P < 0.01$) in DENV-1-injected ($P < 0.03$) and diluent-injected mosquitoes ($P < 0.01$) when compared to the uninjected cohorts, but no difference was seen between DENV-1-injected and diluent-injected mosquitoes (Figure 11).

DENV-1 Viral RNA in the Head of Responder and Nonresponder Mosquitoes on Different Days Post Injection when Exposed to 0.14% DEET

Mosquitoes that displayed irritancy and escaped to the clear chamber when exposed to DEET are categorized as responder, while those that did not display irritancy and just stayed in the metal chamber when exposed to DEET were categorized as nonresponder. DENV-1 RNA was detectable in the mosquito heads starting on 1 dpi. The viral RNA increased significantly each dpi in both responder and nonresponder groups before they plateaued at 14 dpi ($F = 580.62$; df = 5,167; $P < 0.01$). There was no significant difference in the $\log_{10}$ viral RNA in the heads of responder and nonresponder mosquito groups on any dpi tested ($F = 0.02$; df = 1,167; $P = 0.88$) (Figure 12).

DISCUSSION

Our results indicated that there was no effect of DENV-1 infection on *Ae. aegypti*'s behavioral response towards DEET in a contact irritancy bioassay. All three *Ae. aegypti* test cohorts (DENV-1-injected, diluent-injected, and uninjected mosquitoes) demonstrated a significant irritancy response against exposure to 0.14% and 2.5% DEET, which indicates full sensitivity to DEET and viability of both chemical and methodology for study evaluations. Our analysis suggests that there is no significant behavioral difference among all DENV-1-injected, diluent-injected, and uninjected mosquito cohorts when exposed to 2.5% DEET. On the other hand, results from 0.14% DEET exposure showed that there were differences between DENV-1 injected and uninjected cohorts and also between diluent-injected and uninjected cohorts on 10 dpi, but not between DENV-1 and diluent-injected cohorts. The later result would have signified an effect of DENV-1 infection on mosquito behavioral response towards DEET. However, these differences were only observed on a single day with a single concentration of DEET. Therefore, we believe that it is too premature to use this result as evidence that the intrathoracic inoculation is the plausible cause of the behavioral change. Had it been the intrathoracic inoculation process that caused the behavioral change, we would have expected the change to occur on multiple dpi and at both doses. Despite the relatively high morbidity observed in the mosquitoes exposed to DEET, neither DENV infection nor inoculation affected mosquito mortality when exposed to DEET.

Our results are in contrast with the findings of Qualls et al. (100-102) that showed reduced aversion to DEET in *Ae. aegypti* mosquitoes with disseminated SINV infection. In addition, Rift Valley fever virus-infected *Cx. tarsalis* (Coquillett) and *Ae. taeniorhynchus* (Wiedemann) also responded differently to DEET treated hamsters than

negative controls of the same species (unpublished data). This may be explained by the different genera of arboviruses used in the various studies, as members of the genus *Alphavirus* (e.g. eastern equine encephalitis virus, CHIKV, SINV) tend to replicate to higher titers in their arthropod host and to induce higher mosquito host mortality compared to flaviviruses (e.g. DENV, YFV, JEV, and Zika virus) (75). As higher mortality is usually associated with increased damage or change in the host, it might explain why our results are in contrast with the DEET studies using SINV-infected *Ae. aegypti* (100-102). Moreover, those studies were designed to measure the true repellency effect of DEET by providing a sugar or blood source that had been treated with DEET, while our study measured the irritancy effect of DEET (Qualls et al. 2011, 2012a, b). However, our results are in agreement with Frances *et al*. who did not observe any behavioral response differences when intrathoracically DENV-infected mosquitoes were exposed to 5% DEET (38).

As has been previously published by Grieco *et al*., chemicals used in vector control can have singular or multiple functions that can be classified as irritant, repellent, and toxicant (43). Both irritants and repellents can deter insects from biting. However, irritants require contact between the insect and treated surface while a repellent does not (23; 43). Although a toxicant may kill the insect, it may not do so rapidly enough to prevent it from biting. Even though it is well known as a repellent, DEET may possess multiple characteristics of a vector control chemical (137). Our study only examined changes in an irritancy response of *Ae. aegypti* to DEET following DENV-1 infection using the HITTS chambers (44) As such, it is necessary to also assess if the spatial repellent activity of DEET can be altered by DENV-infection. Nevertheless, considering

the level of DENV RNA in the *Ae. aegypti* mosquito nervous system, it is interesting that the irritancy behavior was not altered.

In conclusion, our study indicates that there was no biological difference in the behavioral response of DENV-1-infected or uninfected mosquitoes against DEET. As most, if not all, the DEET insect repellent being marketed have higher concentrations than 2.5%, our study provides evidence to suggest the viability of DEET to be continually used as a personal protective measure against DENV infection. However, further studies evaluating mosquito infection with other DENV serotypes and/or strains, other arbovirus infections, or other insect repellents on the behaviors of disease vectors are warranted in order to provide better recommendation on the prevention of vector-borne disease transmission.

ACKNOWLEDGEMENTS

We would like to thank our colleagues at the Entomology and the Viral Diseases Branches at the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD, especially E. Rowton, J. Richardson, S. Davidson, and T. Rowland for the use of the WRAIR containment facility, providing *Ae. aegypti* mosquito populations, and the DENV-1 stock. We would also like to thank K. Chauhan of USDA for providing neat-grade DEET and W. Ching and C. Chao of Naval Medical Research Center for their generosity in lending their Applied Biosystem 7500 Fast RealTime PCR Machine. This study was supported by the USUHS grants no. R08701 and T0872211113.

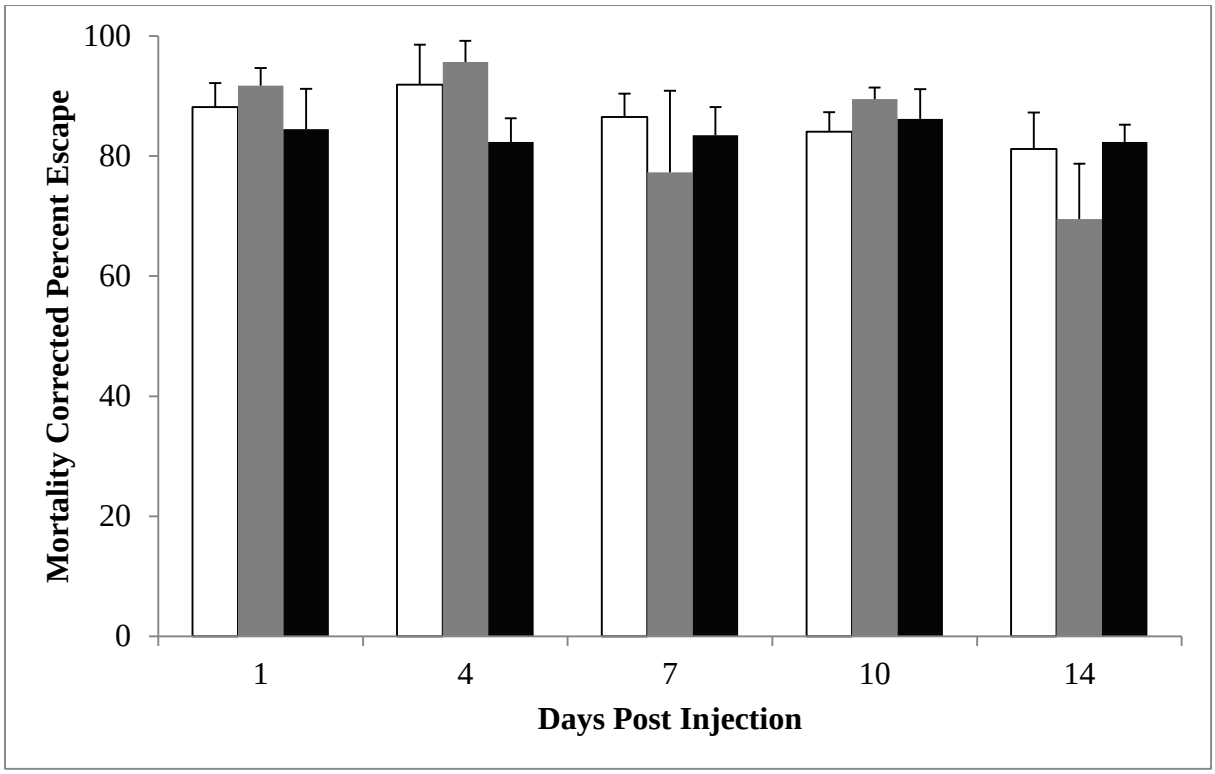


Figure 10. Contact irritancy (escape) response against 2.5% DEET on various days post injection. (□) DENV-1-injected *Ae. aegypti*, (▒) diluent-injected *Ae. aegypti*, (■) uninjected *Ae. aegypti*.

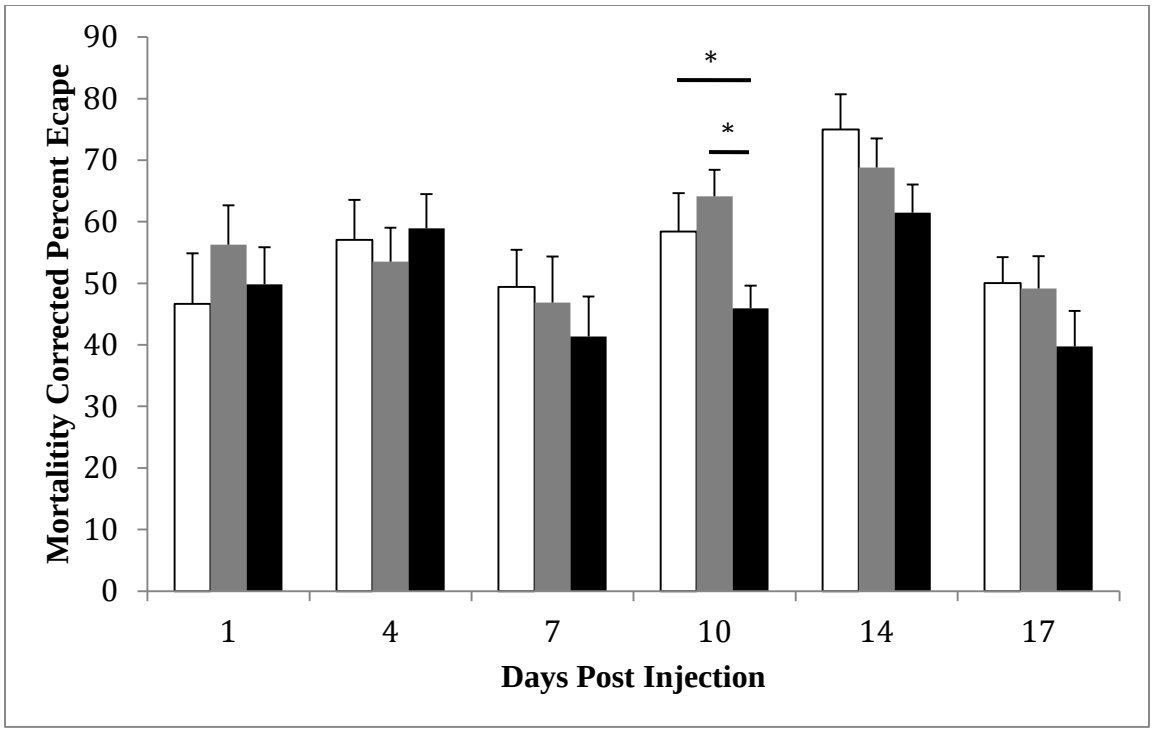


Figure 11. Contact irritancy (escape) response against 0.14% DEET on various days post injection. (□) DENV-1-injected *Ae. aegypti*, (▒) diluent-injected *Ae. aegypti*, (■) uninjected *Ae. aegypti*.

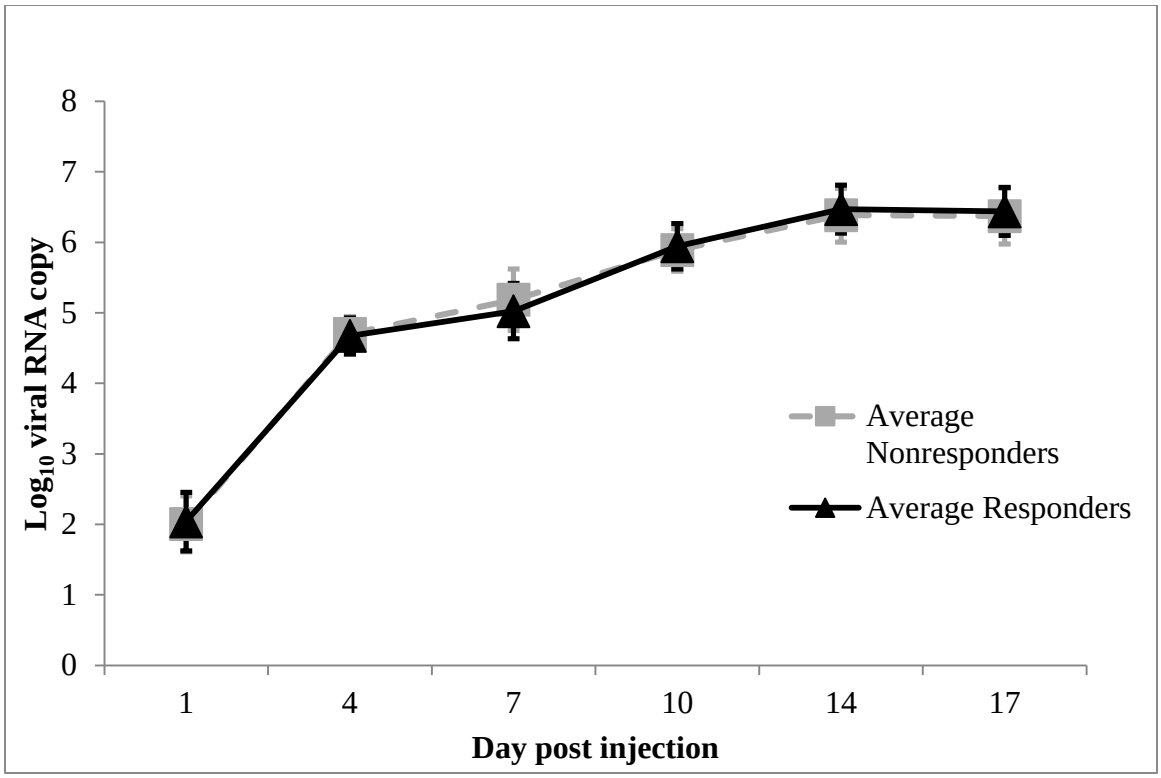


Figure 12. The average log_{10} DENV-1 viral RNA in the head of responder and nonresponder mosquitoes on different days post injection when exposed to 0.14% DEET (N= 30).

Chapter 3: Effects of Pre-exposure to DEET on the Downstream Blood-feeding Behaviors of *Aedes aegypti* Mosquitoes

Submitted as: Sugiharto VA, Grieco JP, Murphy JR, Olsen CH, Colacicco-Mayhugh MG, Stewart VA, Achee NL, Turell MJ to the Journal of Medical Entomology (submission number: JME-2016-0020).

Sugiharto VA designed and conducted the experiments, analyzed the data, and wrote the manuscript.

Grieco JP designed the experiments and edited the manuscript.

Murphy JR analyzed the data and edited the manuscript.

Olsen CH designed the experiments, analyzed the data, and edited the manuscript.

Stewart VA analyzed the data and edited the manuscript.

Colacicco-Mayhugh MG analyzed the data and edited the manuscript.

Achee NL designed the experiments and edited the manuscript.

Turell MJ designed the experiments, analyzed the data, and edited the manuscript.

ABSTRACT

Mosquito behavior is heavily influenced by the chemical molecules in the environment. Modifying insect behavior by harnessing this knowledge as a means to reduce vector-host contact is a powerful method for disease prevention. N,N-Diethyl-meta-toluamide (DEET) is the most widely used insect repellent on the market and an excellent example of a chemical that has been used to modify insect behavior for disease

prevention. However, genetic insensitivity and habituation in *Aedes aegypti* (L.) mosquitoes after pre-exposure to DEET have been reported. In this study, we investigated the temporal effect of pre-exposure to DEET on the downstream blood-feeding behavior of *Ae. aegypti* mosquitoes. We exposed mosquitoes to four different DEET concentrations: 0.10, 0.12, 0.14, and 0.16% for 10 minutes, and then allowed the mosquitoes to blood-feed on an artificial blood-feeding system either immediately or after being held for 1, 3, 6, or 24 hours following DEET exposure. We found that pre-exposing *Ae. aegypti* mosquitoes to 0.14 or 0.16% DEET lowered their blood engorgement level, but did not alter their landing and probing behavior as compared to the control test populations. The reduction in complete blood-feeding was observed at all time periods tested, but was only statistically significant at 3 and 6 hours after the pre-exposure process. Because reduction in blood meal has been associated with increased refeeding, future studies analyzing the effect of this behavior using arbovirus-infected mosquitoes are needed to address the concern of potentially increased vectorial capacity.
Keywords: *Aedes aegypti*, DEET, behavior, blood-feeding

INTRODUCTION

Mosquito behavior is heavily influenced by chemical molecules in the environment that either attract or repel the mosquitoes. The maxillary palps and antennae are the main sensory organs of the mosquito and contain abundant sensilla that house the odorant receptor neurons (3; 55; 93). The insect olfactory process starts when odorant molecules enter the pores located on the sensilla. Each sensillum contains olfactory receptor neurons that have odorant receptors on the surface. As the molecule enters the pores, the odorant binding protein binds and solubilizes the molecule to be transported to

the dendrite of the neuron. The odorant molecule is then recognized by the appropriate odorant receptor and triggers the activation of the olfactory neuron. The activated neuron transfers the information to the corresponding glomeruli in the antennal lobe of the brain that further delivers the signal to the mushroom body of the brain, which processes learning and memory, and brain lateral horn, which directs innate behaviors (48; 124; 132).

As insect bites are not only a source of nuisance, but also can spread disease causing organisms, the WHO believes that reducing the contact between vectors and human hosts is a very powerful method to prevent infection (139). Vector control chemicals possess three different modes of action: repellent (deter biting without direct contact), irritant (deter biting, but requires contact between vector and treated surface), and toxicant (kills the vector (23; 43). The use of topical insect repellent is a preventive measure that is widely available and used to reduce host-vector interaction (20; 49). DEET is an example of a topical insect repellent that has been on the market for almost 70 years. Due to its cost, effectiveness, and safety, DEET is one of the most widely used insect repellents available (137). In addition to its repellent activity, DEET also has been demonstrated to have irritant and toxic effects (80). The Centers for Disease Control and Prevention guidelines for DEET application mention that concentrations <10% confers 1-2 hours of protection and the efficacy plateaus after 50% (20). Longer protection can be achieved by using different DEET formulations or chemical carriers (65).

Unfortunately, insect insensitivity to DEET has been reported (95; 123). Previous studies have shown that insensitivity to DEET seemed to be a genetic trait (95; 123). One study showed that even though there is a genetic determinant that causes insensitivity to

DEET, *Aedes aegypti* (L.) mosquitoes can also "learn" to avoid DEET after initial exposure. Moreover, the can become less sensitive upon subsequent exposures (122). That study showed that mosquitoes that have previously been exposed to 20% DEET, regardless of whether the DEET was applied to a human arm or only a heat source, would become less sensitive when they were exposed to DEET 3 hours after the initial exposure. Another study, that included more odorants, showed similar results and found that habituation did not occur with all tested chemicals (135). These studies demonstrate that previous exposure to DEET may alter the behavior of exposed mosquitoes.

Because of the relatively long protection that can be provided by DEET and the nonuniformity of DEET application in the population, it is important to take a step back and observe if pre-exposure to DEET can alter the most epidemiologically important aspect of mosquito behavior - their blood-feeding behavior. Our study aim was to assess the effect of DEET pre-exposure on the landing and probing behaviors and the engorgement levels of *Aedes aegypti* mosquitoes after pre-exposure to DEET.

MATERIALS AND METHODS

Mosquito Rearing

Aedes aegypti mosquitoes (Belize strain ≤F5) were reared according to standard laboratory procedures. The colony was derived from wild-caught larvae (P1) from Orange Walk Town, Belize (18°04.938'N, 88°33.390'W) in 2014. Egg strips were soaked in water and vacuumed for 1 hour to help synchronize the hatching process. The larvae were fed with Cichlid Gold ™ fish food (Hikari USA, Hayward, CA). All mosquitoes were maintained at 28°C with 80% humidity and a 12 hours light-dark cycle. After molting into second instar larvae, they were separated into groups of 50 larvae in

individual 450 ml plastic cups. After approximately 7 days, pupae were manually sorted into groups of 250 female and placed in 3.9-liter bucket cages (white plastic bucket with attached sleeve for mosquito collection access). At 4-5 days post eclosion, adult female mosquitoes were provided with a 10% sugar solution (Duncraft Inc., Concord, NH) in water from soaked cotton balls and were starved for 24 hours before the assay. For assays where the mosquitoes were held for 24 hours after exposure to DEET, they were provided with a water soaked cotton pad.

Exposure Assay

DEET Pre-exposure

Thirty female *Ae. aegypti* mosquitoes were pre-exposed to DEET (treatment cohort) in a high throughput screening system (HITSS) chamber for 10 minutes and 30 females were pre-exposed to ethanol (control cohort) in another chamber as a control for 10 minutes (44). The chamber is a metal cylinder with a smaller cylinder metal insert where material that has been pretreated with the chemical of choice can be attached to it with magnets. Both ends of the chamber are secured with removable plastic caps equipped with dental dam-gated holes for introducing the mosquitoes into the chambers. The DEET concentrations that were used in this study were 0.10, 0.12, 0.14, and 0.16% diluted in ethanol from a 5% DEET stock solution (USDA, Beltsville, MD). Those concentrations were chosen based on the dose-response curve obtained from a DENV-*Ae. aegypti* behavior experiments (unpublished data). An additional experiment using 5% DEET, but with only a 1 minute exposure was also conducted to assess the effect of a higher concentration of DEET on short-term exposure.

Holding System

We tested five different holding periods: immediately after exposure/no holding time (T0) and after 1 (T1), 3 (T3), 6 (T6), and 24 hours (T24) post-exposure (Figure 13). After pre-exposure, the mosquitoes were collected using a mechanical aspirator and placed into holding tubes (plastic tube with mesh screen on the bottom and open-ended tops that were closed with rubber caps). T0 mosquitoes were immediately used for testing. For the other holding times, the mosquitoes were transferred into pint cups and incubated at 28^{o}C with 80% humidity until tested. Once the holding times were over, the mosquitoes were aspirated from the pint cups into the holding tubes. The holding tubes containing mosquitoes were given to a third party who randomly labeled them in order to reduce bias in data recording by the personnel conducting the experiments.

Post-exposure Blood-feeding Behavior Observation

To observe the blood-feeding behavior, 20 mosquitoes from each test population were put into separate Plexiglas® boxes with an artificial blood-feeding system placed on top of the box (Figure 14). The mosquitoes were allowed to blood-feed for 20 minutes. The landing, probing, and engorgement behaviors were observed. The observation for landing and probing were conducted at 30 seconds intervals for the first 5 minutes. Landing was defined as the number of mosquitoes on the blood source, while probing was defined as the number of mosquitoes probing or feeding at the blood source. At the end of 20 minutes, the mosquitoes were collected, put in the freezer (-20^{o}C) to knock them down, and then graded for engorgement according to the method by Pilitt and Jones (96). The observers were blinded as to the status of the test cohorts. Six replicates were conducted to obtain necessary statistical power with the observers switching the box that they observed after three replicates.

Data Analysis

The number of mosquitoes landing and probing were compared between the treatment and control cohorts using the Mann-Whitney U test (SPSS 22.0 software IBM Corp., Armonk, NY). The corresponding effect size for landing and probing was summarized using the rank-biserial correlation, which measured the strength of association between condition and number of landings/probings. Values close to 0 indicated minimal difference between cohorts, and values close to 1 indicated maximal difference between treatment cohorts. Statistical analysis of landing and probing experiments is based on a sample size of 6 replicates per condition. The blood engorgement level data were combined into two groups: 0-3 (no to little blood-feeding) and 4-5 (engorged) then analyzed with the Fisher's exact test with two-tailed *P*-value using GraphPad (GraphPad Software, La Jolla, CA) and Stepdown-Sidak post hoc analysis on Microsoft Excel 2010 (Microsoft Corp., Albuquerque, NM). Statistical analysis of blood engorgement is based on a sample size of 120 mosquitoes per condition.

RESULTS

Landing and Probing

The effect of DEET on the landing and probing behaviors of *Ae. aegypti* mosquitoes was determined by comparing the landing and probing rates between mosquitoes that were pre-exposed to DEET (treatment cohort) and ethanol (control cohort) (Table 1). No statistically significant difference was observed between the treatment and control populations at any concentration of DEET used in the pre-exposure at any given incubation time. This suggested that pre-exposure to DEET did not affect landing or probing behavior ($P = 0.10$ for both landing and probing).

Engorgement

To examine the effect of DEET on blood engorgement levels in *Ae. aegypti* mosquitoes, we combined the data from the replicates at each time period for each exposure cohort. We then compared the number of mosquitoes with no to moderate engorgement (grades 0-3) with those that took a nearly complete blood meal (grades 4 and 5). This was done for mosquitoes that were pre-exposed to DEET or ethanol (control) (Table 2). No statistically significant difference was observed when the mosquitoes were given a blood source immediately after the DEET exposure process (T0) at any concentration. Therefore, for subsequent experiments, the lowest DEET concentration (0.10%) was not tested further. Similarly, when tested after 1 hour incubation (T1), the engorgement level of mosquitoes that were pre-exposed to any concentration of DEET did not show any statistical significant difference. Therefore, in the subsequent experiments, 0.12% DEET was also eliminated.

The engorgement level of mosquitoes that were pre-exposed to 0.14 or 0.16% DEET were reduced when compared to the control once incubated for 3 or 6 hours after the pre-exposure step ($P \leq 0.02$). This reduction of blood engorgement level was still detectable at 24 hours but it was no longer statistically significant ($P = 0.38$). Overall, the blood engorgement level was statistically reduced within 24 hours after pre-exposure to 0.14 or 0.16% DEET ($P < 0.01$).

Pre-exposure to High DEET Concentration

As DEET is marketed at higher concentrations than what we tested, we conducted an additional experiment to see if exposure to a higher concentration, but for a shorter time, would produce a similar result as the previous experiments (lower concentration at

relatively longer time). All three observed behaviors; landing, probing, and engorgement levels; were not significantly different between mosquitoes pre-exposed to 5% DEET and mosquitoes pre-exposed to ethanol when they were tested 6 hours after they had been exposed for 1 minute (landing and probing $P = 0.087$; blood engorgement level $P = 0.088$).

DISCUSSION

We found that pre-exposure to DEET may alter certain aspect of *Ae. aegypti* blood-feeding behavior. Mosquitoes that had been pre-exposed to DEET did not show any difference in their landing and probing rates compared to the control cohorts. However, we observed reduced blood engorgement level in *Ae. aegypti* mosquitoes that were pre-exposed to DEET. The significant reduction in engorgement level was observed overall within 24 hours after 0.14 or 0.16% DEET exposure, but especially significant and occurred consistently at 3 and 6 hours after pre-exposure. These findings may have implications on the mosquito vectorial capacity because reduction in blood engorgement level has been associated with increased refeeding (34).

In contrast, no change in blood-feeding behavior was observed when the mosquitoes were tested immediately after DEET exposure. While this seems counterintuitive, we speculate that when the mosquitoes were tested immediately, they did not have sufficient time to recover from handling during the pre-exposure process. Thus blood-feeding was likely inhibited in both cohorts, masking the effect of DEET. Moreover, as the incubation period increased up to 6 hours after exposure, the mosquitoes became hungrier and fed better on the blood source.

Even though we did not see any changes in the landing and probing rates of the mosquitoes between those pre-exposed to DEET and ethanol, the differences in engorgement levels at the end of the 20-minutes blood-feeding period suggest that there may have been differences that were not observed. These may have been missed because landing and engorgement behaviors were only observed during the first 5 minutes of the blood-feeding observation.

The level of DEET that evaporates from or is absorbed by the skin varies greatly depending on the initial concentration applied, how long it has been applied, and the formulation of the DEET solution (35; 65; 107). This existing variation makes it very difficult, if not impossible, to ascertain specific concentrations to be tested. After we saw that mosquitoes pre-exposed to 0.14 or 0.16% DEET for 10 minutes displayed significantly less engorgement, we tested a higher concentration of DEET (5%), but for a shorter time (1 minute). Interestingly, no behavioral changes were observed in the landing, probing, or engorgement levels in the mosquitoes pre-exposed to 5% DEET for 1 minute.

Although mosquitoes pre-exposed to DEET were still at least 3-fold less likely to obtain a complete blood meal at 24 hours as those pre-exposed to ethanol, these differences were no longer statistically significant. The lack of statistical significance may be due to the relatively poor feeding in the mosquitoes held for 24 hours. In the studies conducted at 24 hours, the control mosquitoes were significantly less likely to obtain a complete blood meal than those tested at 3 or 6 hours ($P \leq 0.01$), making it more difficult to obtain statistical significance despite the >3-fold difference in feeding success. The reduced feeding might have been because these mosquitoes had been provided with a

water source that might have increased their satiety levels. Overall, we found that *Ae. aegypti* mosquitoes that had been exposed to 0.14% or 0.16% DEET exhibited reduced blood engorgement levels compared to the control cohort within 24 hours after exposure ($P < 0.001$), with the highest difference occurring at 3 and 6 hours after the exposure process.

It is important to note that had the mosquitoes in the combined category of 0-3 been mostly those that did not obtain any blood meal at all (category 0), this would have been a good thing. That finding would mean that prior exposure to DEET would cause the mosquitoes to be less likely to feed within 24 hours from exposure. Unfortunately, that was not the case. Most of the mosquitoes in this study took partial blood meals with only 4.7% of them in category 0. Furthermore, no significant difference was observed in the number of mosquitoes in category 0 between those that were exposed to DEET or control ($P \geq 0.2$). Thus, the mosquitoes with prior exposure to DEET would be less likely to obtain a complete blood meal and would be more likely to take multiple blood meals, potentially becoming more efficient vectors.

Aedes aegypti mosquitoes can transmit many arboviruses, such as: DENV, CHIKV, YFV, or Zika viruses. As noted above, if these mosquitoes were infected with an arbovirus and then were repelled by DEET, they might not feed to engorgement on their next host, thus prompting them to bite more often on more hosts, further spreading the virus. Moreover, a previous study also indicated that pre-exposure to DEET reduced the mosquito repellency to the subsequent DEET exposure; the repellent was less effective at protecting against mosquito bites (122). Furthermore, it will be interesting to see if

similar behavioral changes also happen in other disease vectors such as *Anopheles* mosquito, sand flies, and kissing bugs.

In conclusion, even though DEET has been used for decades, there are still many aspects of DEET use that still need to be better understood. Additionally, even though there is a growing body of evidence that chemical pre-exposure can alter the subsequent behavior of other insects that are vectors for diseases, the number of research studies in this area is still lacking. The possibility of DEET pre-exposure causing higher incidence of arboviral infection is of particular concern because DEET application is a part of the greater efforts at reducing vector and host contact as a means to reduce the incidence of the disease. Further research using arbovirus-infected mosquitoes is necessary to address this concern. Expansion of the scope of similar behavioral alteration research to other diseases vectors may help us better utilize the tools we have in the fight against vector-borne diseases.

ACKNOWLEDGEMENT

The authors would like to thank W. DeYoung of USUHS for his assistance in the experiments. We also thank E. Atkins, A. Caranci, S. Polsomboon, W. Thipmontree, V. Raman, C. Kifude, R. Miller, E. Parsons, and D. Stiffler, all at USUHS, for their assistance in the blinding process. Our appreciation also goes to K. Chauhan of the United States Department of Agriculture for providing DEET.

Table 1. The rank-biserial correlation of landing and probing behaviors between DEET pre-exposed and control mosquitoes.

Incubation time	DEET concentration							
	0.10%		0.12%		0.14%		0.16%	
	Landing	P-value	Landing	P-value	Landing	P-value	Landing	P-value
T0	0.39	0.26	0.22	0.48	0.03	0.90	0.06	0.87
T1	N/A[a]	N/A	0.28	0.42	0.42	0.21	0.56	0.10
T3	N/A	N/A	N/A	N/A	0.36	0.30	0.31	0.38
T6	N/A	N/A	N/A	N/A	0.19	0.57	0.42	0.23
T24	N/A	N/A	N/A	N/A	0.33	0.14	0.33	0.14
	Probing	P-value	Probing	P-value	Probing	P-value	Probing	P-value
T0	0.42	0.22	0.17	0.53	0.03	0.90	0.06	0.87
T1	N/A	N/A	0.17	0.63	0.17	0.62	0.56	0.10
T3	N/A	N/A	N/A	N/A	0.42	0.23	0.44	0.20
T6	N/A	N/A	N/A	N/A	0.19	0.57	0.42	0.23
T24	N/A	N/A	N/A	N/A	0.33	0.14	0.33	0.14

[a]N/A = If in the previous time point experiments none of the blood-feeding behaviors were statistically significantly different, then the concentration was not used for the subsequent holding time experiments.

Table 2. Blood engorgement level between DEET pre-exposed or control mosquitoes.

Incubation time	DEET concentration	Cohort	Blood engorgement level[a]		P-value	
			0-3	4-5	Fisher's exact test[b]	Stepdown-Sidak[c]
T0	0.10%	Treatment	105	12	0.68	0.97
		Control	103	15		
	0.12%	Treatment	112	5	1	1
		Control	112	6		
	0.14%	Treatment	120	2	0.1	0.46
		Control	110	7		
	0.16%	Treatment	109	6	0.6	0.97
		Control	111	9		
T1	0.12%	Treatment	113	10	0.11	0.43
		Control	120	4		
	0.14%	Treatment	99	14	0.69	0.9
		Control	110	13		
	0.16%	Treatment	108	12	0.06	0.13
		Control	99	28		
T3	0.14%	Treatment	111	7	0.001	0.01
		Control	101	25		
	0.16%	Treatment	106	14	0.0004	0.005
		Control	84	37		

T6	0.14%	Treatment	110	12	0.002	0.02
		Control	87	30		
	0.16%	Treatment	105	13	<0.0001	0.001
		Control	68	54		
T24	0.14%	Treatment	113	5	0.03	0.24
		Control	105	15		
	0.16%	Treatment	119	1	0.07	0.38
		Control	116	7		

[a] Engorgement grades according to the method by Pilitt and Jones (Pilitt and Jones 1972).
[b] As determined by two-tailed Fisher's exact test with without correction.
[c] *P*-values after Stepdown-Sidak post hoc analysis/correction.

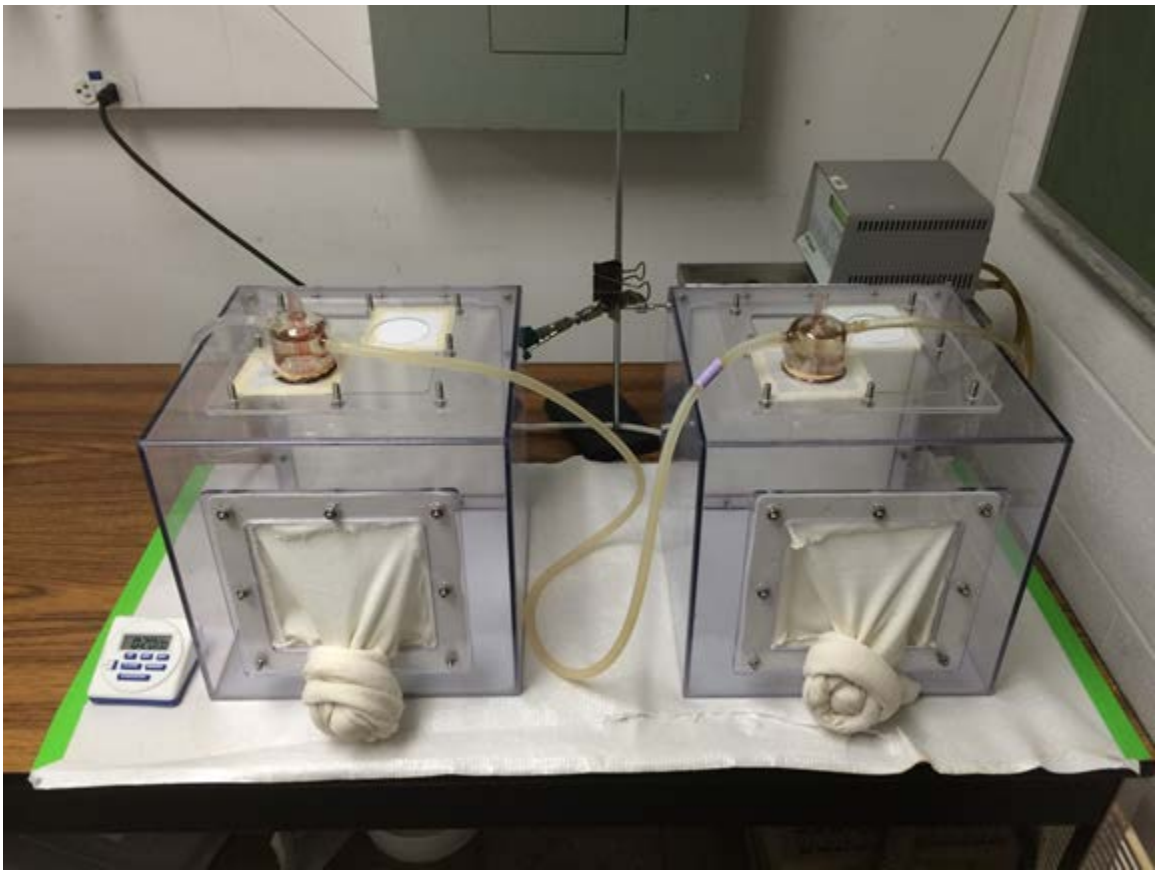


Figure 13. Plexiglas® box set up for blood-feeding behavior observation.

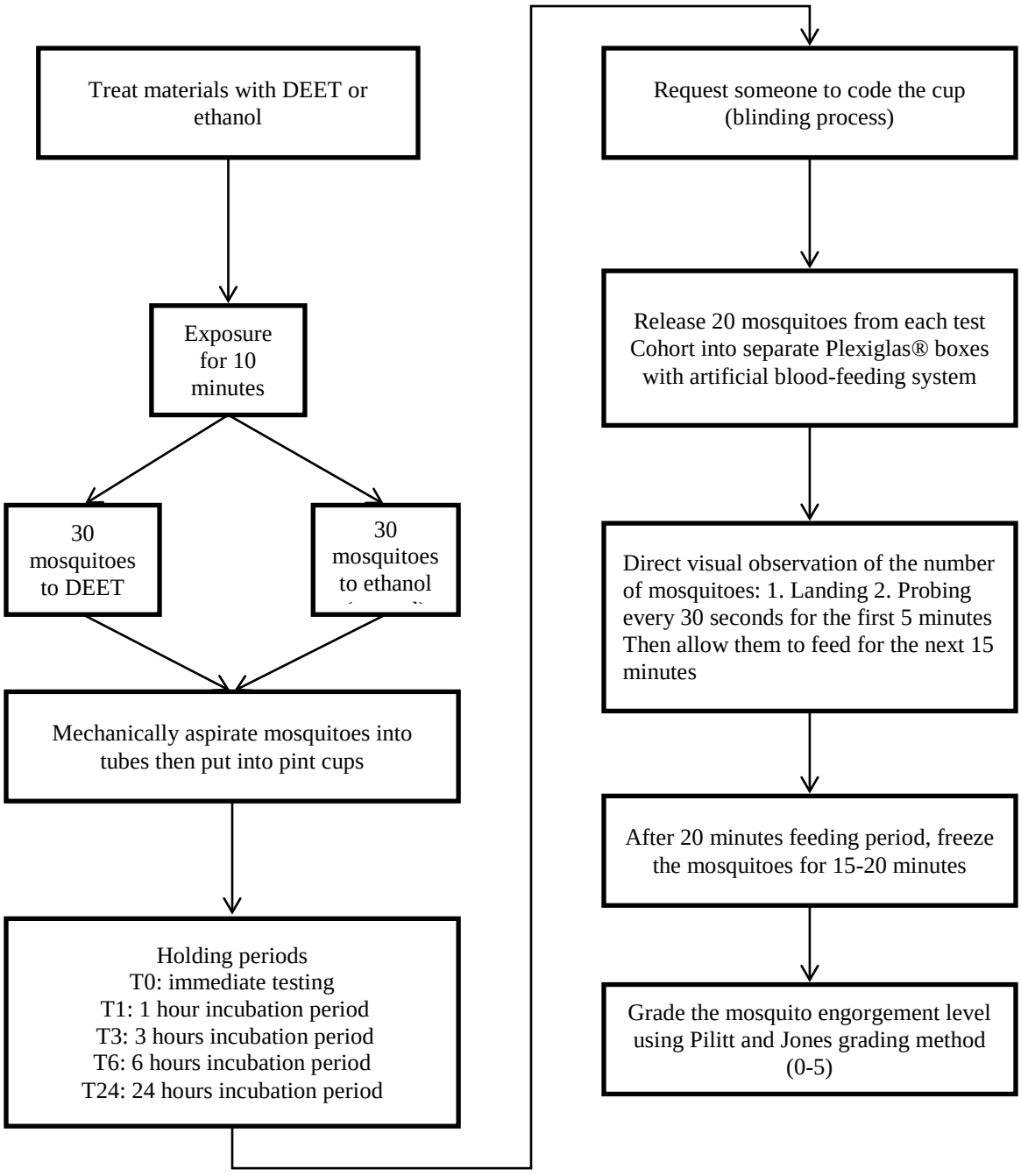


Figure 14. Experimental study design.

CHAPTER 4: General Discussion And Conclusion

GENERAL DISCUSSION

Aedes aegypti remains a big threat to global health as it continues to spread diseases such as DENV, which are not only debilitating but also kill millions of people each year. After rigorous research into the development of a DENV vaccines, the Sanofi Pasteur DENV vaccine Dengvaxia® was just licensed in Mexico, the Philippines, and Brazil albeit with varying effectiveness (36; 103; 116). So far, there is limited availability and data on the vaccine. However, it is a great addition to the public health tools we have for fighting DENV. That being said, reducing vector-host contact is still needed in conjunction with the DENV vaccine. More importantly, for many other vector-borne diseases whose vaccines have not been developed yet (e.g., chikungunya and Zika), reducing contact is still the best prevention method available.

The lack of information on the mosquito olfactory system as a means to completely understand what drives mosquito behavior causes delays in the development of new repellent chemicals. Therefore, we are limited to the use of existing preventive tools as a means to reduce vector and host contacts. One of the best available repellent compounds is DEET. Unfortunately in recent years, there have been reports of insect insensitivity to DEET. Additionally, there have been reports on *Ae. aegypti* mosquito behavior alteration following infection or previous exposure to chemicals. These changes could possibly reduced the overall effectiveness of DEET. The overall goals of this project were to observe if:

1. Dengue infection can alter the *Ae. aegypti* mosquito behavior towards DEET.

2. Pre-exposure of *Ae. aegypti* mosquitoes to DEET can change their downstream blood-feeding behaviors.

Exploring the Effect of Dengue Virus Infection on *Aedes Aegypti* Behavioral Response to DEET

Research on new chemicals geared towards reducing vector-host contact is often tested using uninfected mosquitoes. This type of research also focuses heavily on the chemical's ability to kill mosquitoes and less on the ability to deter mosquitoes via irritancy or repellency. Fortunately, more and more attention has been given to the irritancy and repellency effect of these chemicals, which usually appear in sub-lethal dose exposures (43). Interestingly, several studies have shown that arbovirus infection can alter mosquito's response towards repellent and may potentially reduce effectiveness in the field (100-102). This information adds another layer of complexity concerning advocating the use of repellent as a blanket public health tool.

In Chapter 2, we used the high-throughput screening system (HITTS), which has been used as a tool to analyze the toxicity, irritancy, and spatial repellency activity of chemicals (44). In this experiment, we utilized the system to assess if DENV-1 infection could alter the *Ae. aegypti* mosquito irritancy behavioral response towards DEET. In order to assess any potential association between infected organs and behavioral changes, we conducted the experiments on different days after the virus had been injected into mosquitoes. Our experiments showed no behavioral response alteration among the uninjected control, diluent-injected, and DENV-1 injected *Ae. aegypti* mosquitoes. This result is in accordance with the results of Frances et al. (38) that used the same mosquito species and DENV. However, it is different from studies by Qualls et al. that used the same mosquito species but a different arbovirus (SINV) (100-102). Because we did not

find that DENV-1 caused different behavioral responses to DEET, further study to investigate the molecular aspect that might underlie the behavioral response was not conducted. Our failure to document a difference in DEET response in DENV-infected mosquitoes is exciting because it showed that DEET was still efficacious in causing mosquitoes to be irritated after exposure and thus in reducing vector-host contact.

Our study only investigated the irritancy response of DENV-1-infected *Ae. aegypti* mosquitoes against DEET. Although no statistically significant behavior change was observed in the irritancy behavior, this is not a guarantee that the same result will occur when the spatial repellency response is tested; these two behaviors seem to utilize different receptors (64; 125). In our study, we only focused on the DENV-1 infection as a prototype for investigating behavioral change in infected *Ae. aegypti* mosquitoes. Other DENV serotypes exist and some, like DENV-2, have been associated with more severe clinical outcomes. These might translate to a higher level of damage in the mosquito vector that could ultimately affect its behavior (129). Also, because damage and the infection of the mosquito brain is suspected as the cause for the behavioral change in infected mosquitoes, it may also be a good idea to do a similar study on arboviruses that have a more neurotropic inclination in the vertebrate host (75; 102).

Even though our result showed no significant behavioral alteration response towards DEET in DENV-1 infected *Ae. aegypti* mosquitoes, we did not implicate this result as a reason to bypass the chemical testing with infected mosquitoes. Indeed, this result actually created a need for further research; different arboviruses seem to cause a variety of different behavioral responses toward DEET. Further experiments using different DENV serotypes, arboviruses, or potential vectors, such as *Ae. albopictus*, are

necessary to ensure that the public health tool being deployed to combat these diseases caused by mosquito-borne pathogens are utilized to their utmost potential. As most of the mosquito-borne pathogens are prevalent in developing countries, these future studies may highlight the need for more customized prevention tools that are more sustainable and cost-effective. Such tools could substantially reduce the burden on the public health infrastructure of the country.

The Effects of Pre-exposure to DEET on the Downstream Blood-feeding Behaviors of *Aedes Aegypti* Mosquito

The insect olfactory system is very complex and the exact mechanism of how DEET deters mosquitoes from biting is still not fully understood. Several studies have shown that pre-exposure to DEET can alter insects' subsequent behavioral response towards that chemical by reducing the insect sensitivity to it in a so-called habituation process (122; 135). Because mosquitoes continue to seek a blood meal after being repelled, DEET is not uniformly applied in the population, and DEET effectiveness decreases overtime, it is very common to find a mosquito that was previously exposed to DEET at any given time. However, how this subset of the mosquito population behaves has not been investigated. As blood-feeding is the most epidemiologically important mosquito behavior, we decided to investigate the landing, probing, and blood engorgement level aspects of this behavior. To address this question, as outlined in Chapter 3, we exposed *Ae. aegypti* mosquitoes (originally from Belize, $\leq$ F5) to DEET. In contrast to the strain of *Ae. aegypti* Liverpool mosquitoes in Chapter 2 that have been maintained for a long time in a laboratory setting, the Belize strain that we used in Chapter 3 were collected from the field and had been maintained in the laboratory for $\underline{\leq}$ 5 generations; thus better retaining their wild-type traits. We used the lowest dose that

could still induce irritancy in the *Ae. aegypti* Liverpool strain in the HITTS chambers as well as several other doses that were lower and higher. After exposing the mosquitoes to DEET, we either immediately allowed them to blood-feed on an artificial blood-feeding system or held them for various length of time to observe the length of behavioral change caused by the pre-exposure process.

Our result in Chapter 3 showed that the landing and probing rates of DEET pre-exposed *Ae. aegypti* mosquitoes were unchanged from the control. However, we observed that the blood engorgement level of these DEET pre-exposed mosquitoes was significantly lower. Specifically we saw more partial blood-feeding, but no changes in the number of no blood-feeders. The reduced blood engorgement level was observable as late as 24 hours, although only 3 and 6 hours post exposure were statistically significant. This result suggests that it is possible that mosquitoes that are pre-exposed to DEET are more likely to reefed; partial blood meal feeding has been associated with refeeding (61). If we connect this result with the result from Chapter 2 where DENV-1 infected mosquitoes did not have any change in behavioral response to DEET, it is possible that these DEET pre-exposed mosquitoes could have a higher vectorial capacity to transmit DENV. In addition, as previously demonstrated by Stanczyk et al., DEET pre-exposed mosquitoes became less susceptible at their subsequent exposure to DEET, which rendered the repellent to be even less effective at reducing vector-host contact in subsequent hosts (122). Nevertheless, we do note that the DEET concentrations that were used in our study were very low with a relatively long duration of exposure. In the real life setting, DEET is sold at higher concentrations and exposure occurs in much shorter periods of time. Preliminary experiment that used 5% DEET with only 1 minute of exposure, did not

show a similar effect as the ones with the lower concentrations and 10 minutes exposure process.

These results are indeed still inconclusive. Many questions arise that will require more studies. Because DEET has multiple modes of action against insects, it would be interesting to study the mechanism of how prior chemical exposure can cause the observed reduction on the blood engorgement level. Also, even though we showed that the blood engorgement level decreased after DEET pre-exposure, we have only tested it using an artificial blood-feeding system. A study using animals as the blood source may be better able to assess if this reduction also exists when host odorant cues come into play.

Interestingly, a previous study had shown that DENV-2 infected mosquitoes also display a reduced blood engorgement level (97). Similar to our study, those investigators speculated whether the DENV-2 infected mosquitoes would have a higher vectorial capacity. It would be interesting to see, if the blood-feeding reduction from pre-exposure to DEET also happens in infected mosquitoes, or if the two effects are synergistic and produce an even greater effect. Indeed, a similar study that allows the mosquitoes to blood-feed at multiple time points may be able to help answer if DEET pre-exposed and infected mosquitoes have higher vectorial capacity than their control counterparts. Even though DEET is the most widely used insect repellent, other repellents exist in the market. It may also be necessary to investigate if any combination of insecticides or repellents has synergistic or antagonistic effects on each other following the pre-exposure process. More fine-tuning on the mosquito withholding time is also needed to see how long this effect actually persists. Lastly, these results adds another insight into how complex the

mosquito olfactory system as well as into many further studies are needed in order to elucidate the complex mechanisms behind the prevention of the vector-borne diseases.

CONCLUDING REMARKS

With limited vaccine availability to combat them, vector control remains the most feasible preventive measure against many vector-borne diseases. Sadly, our understanding of the insect olfactory system, which is the main driver of insect behavior, is still very limited. Despite reports of insect insensitivity to insecticides and repellents, the development of new chemicals also continues to be slow. In addition, our knowledge about the physiological change in mosquitoes that occurs in response to pathogen infection and how these changes affect behaviors is also still very limited.

Our results showed that even though DEET has been around for six decades, many of the properties that are relevant to public health are still unknown. In our initial study, DEET could still induce irritancy behavior in DENV-1-infected *Ae. aegypti* mosquitoes. At first, this seems like a promising result in support of continued DEET application as a means to reduce vector-host contact and reducing DENV infection incidence. However, our second study showed that prior exposure to DEET could potentially cause the mosquitoes to become more dangerous; they may be more inclined to refeed and spread diseases even further. Indeed we did not investigate the interaction between DENV-1 infection and pre-exposure to DEET in this study. However, that study limitation should serve as an indicator of how much work is still needed in this field.

The work presented here hopefully can add more pieces to complete the public health puzzle of vector control. This is specifically true in the field of insect behavior. As shown in our results, there are still many aspects of repellents and vector behaviors that

have not been explored. It may be necessary in the future to not only test repellents against uninfected mosquitoes, but also against infected mosquitoes as a means to determine if pathogen-infected mosquitoes behave differently towards repellent compared to their uninfected counterparts. In addition, further research exploring the blood-feeding behavior of infected mosquitoes that have been previously exposed to repellents is necessary in order to ensure that the usage of repellent does not actually do more harm than good. Because different infections, chemicals, and vectors have different interaction dynamics, we are facing an enormous task in order to answer this problem. As daunting as it is, obtaining this information is necessary for the improvement of public health tools in combating mosquito-borne diseases in a more effective manner.

Appendix A

THE ASSESSMENT OF VIABILITY OF DEET CONCENTRATIONS USED IN THE HITSS IRRITANCY ASSAY

Irritancy assays using HITSS chambers were conducted as previously described in Chapter 2. All three *Ae. aegypti* test populations (DENV-1 injected, diluent-injected, and uninjected mosquitoes) demonstrated significant irritancy against exposure to 0.14 and 2.5% DEET, indicating full sensitivity to DEET and viability of both chemical and methodology for study evaluations (Table 3 and 4).

Table 3. Contact irritancy response against 2.5% DEET (treatment) compared to the ethanol (control chemical).

DPI	Group	Control escape (Mean ± SE)	Treatment escape (Mean ± SE)	*P*-value
1	DENV-1-injected	0.4 ± 0.2	1.8 ± 0.5	<0.01
	Diluent-injected	0.5 ± 0.2	1.3 ± 0.4	<0.01
	Uninjected	0.0 ± 0.0	2.1 ± 0.6	<0.01
4	DENV-1-injected	0.5 ± 0.2	2.4 ± 0.5	<0.01
	Diluent-injected	0.5 ± 0.2	1.5 ± 0.3	<0.01
	Uninjected	1.4 ± 0.6	3.0 ± 0.4	<0.01
7	DENV-1-injected	1.0 ± 0.4	3.2 ± 0.5	<0.01
	Diluent-injected	1.3 ± 0.5	1.9 ± 0.6	<0.01
	Uninjected	0.6 ± 0.3	3.2 ± 0.5	<0.01
10	DENV-1-injected	1.3 ± 0.5	1.3 ± 0.4	<0.01
	Diluent-injected	0.5 ± 0.2	1.7 ± 0.4	<0.01
	Uninjected	0.7 ± 0.3	1.9 ± 0.4	<0.01
14	DENV-1-injected	0.5 ± 0.3	1.5 ± 0.4	<0.01
	Diluent-injected	0.9 ± 0.3	1.8 ± 0.5	<0.01
	Uninjected	0.1 ± 0.1	1.5 ± 0.4	<0.01

Table 4. Contact irritancy response against 0.14% DEET (treatment) compared to the ethanol (control chemical).

DPI	Group	Control escape (Mean ± SE)	Treatment escape (Mean ± SE)	*P*-value
1	DENV-1-injected	0.0 ± 0.0	1.5 ± 0.3	<0.01
	Diluent-injected	0.4 ± 0.2	3.3 ± 0.6	<0.01
	Uninjected	0.3 ± 0.2	2.8 ± 0.6	<0.01
4	DENV-1-injected	0.3 ± 0.1	2.8 ± 0.2	<0.01
	Diluent-injected	0.1 ± 0.1	3.0 ± 0.4	<0.01
	Uninjected	0.5 ± 0.2	4.0 ± 0.6	<0.01
7	DENV-1-injected	0.4 ± 0.2	2.2 ± 0.5	<0.01
	Diluent-injected	0.4 ± 0.3	2.5 ± 0.6	<0.01
	Uninjected	0.3 ± 0.2	2.7 ± 0.4	<0.01
10	DENV-1-injected	0.4 ± 0.2	2.6 ± 0.4	<0.01
	Diluent-injected	1.6 ± 0.4	3.5 ± 0.6	<0.01
	Uninjected	0.4 ± 0.2	2.4 ± 0.3	<0.01
14	DENV-1-injected	0.5 ± 0.2	3.5 ± 0.5	<0.01
	Diluent-injected	0.5 ± 0.3	3.0 ± 0.4	<0.01
	Uninjected	0.4 ± 0.2	3.4 ± 0.5	<0.01
17	DENV-1-injected	0.7 ± 0.3	2.2 ± 0.5	<0.01
	Diluent-injected	0.5 ± 0.1	2.4 ± 0.4	<0.01
	Uninjected	0.3 ± 0.2	2.3 ± 0.5	<0.01

Appendix B

DOSE RESPONSE ANALYSIS

Irritancy assay using HITSS chambers were conducted as previously described in Chapter 2 using various doses of DEET. The dose-response experiments were conducted using an uninjected *Ae aegypti* test cohort. The concentration 0.14% DEET was found to be the lowest dose that could still induce irritancy in *Ae. aegypti* mosquito (Table 5).

Table 5. Contact irritancy response against various doses of DEET.

DEET concentration (%)	Control escape (Mean ± SE)	Treatment escape (Mean ± SE)	*P*-value
1	0 ± 0	4.3 ± 0.7	<0.01
0.7	0 ± 0	4.0 ± 0.6	<0.01
0.4	0 ± 0	3.2 ± 0.7	<0.01
0.2	0 ± 0	3.7 ± 0.3	<0.01
0.18	0.8 ± 0.5	3.5 ± 0.7	<0.01
0.16	0.8 ± 0.5	1.3 ± 0.4	0.03
0.14	0.8 ± 0.5	3.5 ± 0.6	<0.01
0.12	0.8 ± 0.5	2.3 ± 1.1	0.14
0.1	0 ± 0	1.2 ± 0.6	0.06

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